CONNECTIVE TISSUES

Transactions of the Second Conference
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JOSIAH MACY, JR. FOUNDATION CONFERENCE PROGRAM

As AN INTRODUCTION to these Transactions of the Second Conference on Connective Tissues, I would like to outline what it is that the Foundation hopes to accomplish by its Conference Program We are interested, first of all, in furthering knowledge about connective tissues and to this end the participants were brought together to exchange ideas, experiences, data, and methods In addition to this particular goal, however, there is a further, and perhaps more fundamental, aim which is shared by all our conference groups the promotion of meaningful communication between scientific disciplines

The problem of communication between disciplines we feel to be a very real and a very urgent one, the most effective advancement of the whole of science being to a large extent dependent upon it Because of the accelerating rate at which new knowledge is accumulating and because discoveries in one field so often result from information gained in quite another, channels must be established for the most relevant dissemination of this knowledge

The increasing realization that nature itself recognizes no boundaries makes it evident also that the continued isolation of the several branches of science [illegible]
larly [illegible]
discip[illegible]
well versed in nuclear physics because of the tracer techniques and the injury which can result from radiation At the other extreme, medicine is certainly a social science and, through mental health, must be concerned with economic and social questions The answer, then, is not further fragmentation into increasingly isolated specialities, disciplines, and departments, but the integration of science and scientific knowledge for the enrichment of all branches This integration, we feel, can be encouraged by providing opportunities for a multiprofessional approach to given topics

Although the fertility of the multidiscipline approach is recognized, adequate provision is not made for it by our universities, scientific societies, and journals And perhaps the presence of other hindering fac-

tors must be admitted. Partly semantic in nature, they may also to some degree be psychological Admittedly, it is oftentimes difficult to accept data derived from methods with which one is unfamiliar. By making free and informal discussion the central core of our meetings, we hope to achieve an atmosphere which minimizes as much as possible these emotional barriers

Thus, our meetings are in contrast to the usual scientific gatherings They are not designed to present neat solutions to tidy problems but to elicit provocative discussion of the difficulties which are being encountered in research and practice. For this reason, we ask that the presentations be relatively brief and that emphasis be placed on discussion as the heart of the meeting Our hope is that the participants will come prepared not to defend a single point of view but to take advantage of the meeting as an opportunity to speak with representatives of other disciplines in much the same way that they would talk with their own colleagues in their own laboratories.

We have, now, thirteen groups functioning under the Conference Program. The following topics are covered: adrenal cortex, aging, blood clotting, cold injury, connective tissues, consciousness, cybernetics, infancy and childhood, liver injury, metabolic interrelations, nerve impulse, renal function, and shock and circulatory homeostasis

When a new conference is organized, the Chairman, in consultation with the Foundation, selects fifteen scientists to be the nucleus of the group, and every effort is made to include representatives from all pertinent disciplines From time to time new members are added by the group to fill gaps in viewpoint or technique. A limited number of guests are invited to attend each meeting, but, for the purpose of promoting full participation by all members and guests, attendance at any meeting is limited to twenty-five. It is inevitable that in no topic can we possibly include more than a small fraction of the key investigators in the field, and one of the difficulties in forming a group like this is that it is necessary to leave out so many people whom we would like to include.

The transactions of these meetings are recorded and published This is done because the Foundation wishes to make current thinking in a field available to all those working in it, and because it believes that conveying to those in other fields who are concerned with science, for example, government officials, administrators, etc, the essential nature of scientific research is also an important problem in communication. Logic is a [illegible] intuitive or creative as [illegible] portrait or the com[illegible] e, necessary in ord[illegible] rives on

creativity which has its source in unconscious, nonrational processes. Unfortunately, however, in the finished products which are presented to the world through research reports this integral part of scientific endeavor is shriveled by the cold, white light of logic. By preserving the informality of our conferences in the published transactions, we hope to give a truer picture of what actually goes on in the minds of scientists and of the role which creativity plays.

FRANK FREMONT-SMITH, M.D.
Medical Director

SOME FUNCTIONAL CONSIDERATIONS OF GROUND SUBSTANCE OF CONNECTIVE TISSUES

ISIDORE GERSH
Department of Anatomy
University of Chicago School of Medicine

In order to conform with the spirit of the Conference, I have divided the material into eight sections, each of which is a more or less self-contained argument I hope that after I present each section discussion will follow

I would like to introduce the subject of ground substance by placing it in relation to other components of the connective tissues A diagram can be drawn which shows cells in connective tissue, the reticular fibers, a branching elastic fiber, and a collagen bundle * In certain regions, as under the epithelium, and around fat cells, around the blood vessels, and around skeletal muscle cells, the reticular fibers are finer, and also form a closer meshed net.

There is the question of the relationship of these structures to tissue fluid, which is, one might say, one of the nonfibrillar components As can be seen, it is more or less uniformly distributed in the interfibrillar, intercellular space The tissue fluid is related chiefly to hemodynamic factors, to the composition of the plasma, and to capillary permeability

The ground substance may be represented as a homogeneous component, also extracellular, also distributed between the fibrils, so that the fibrils and fibers are immersed in this homogeneous material

In certain places, as under the epithelium and around blood vessels, fat cells, smooth, cardiac, and skeletal muscle cells, the ground substance, as we see it, is different and forms part of the basement membrane Throughout, it is continuous with and inseparable from the homogeneous ground substance of what might be called the loose connective tissue.

Fremont-Smith May I ask a question? Is the ground substance dissolved in the tissue fluid, or is it separate from it? I am not quite clear

Gersh: It is as if you had a red dye in gelatin. The red dye, I suppose, would be dissolved in the aqueous medium, and the gelatin would

*Figures illustrating the material given in this p[illegible] e found in *The Harvey Lec* 1949-1950 Series XLV Springfield, Illinois, Chath[illegible] (p 211). (*In press*)

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be in the gel state or the red dye could be combined with the gelatin in the gel state The tissue fluid and the ground substance are considered to be coextensive and homogeneous, interconnected throughout

Fremont-Smith. If you were to put a needle in and take out the tissue fluid, would you not be taking out ground substance too?

Gersh: In many cases you would

Hill: What do you mean by reticular fibrils?

Gersh: Fibrils which, by definition, are stainable by specific silver stains, and have a structure different from that of collagen and from that of fibrin

Ragan How do you mean different?

Gersh. Do they have the same periodicity and the same structure as collagen?

Porter. As nearly as one can tell.

Angerine: In the young rat they are closely related to collagen and show a gradual transition to collagen with aging (1).

Porter: The silver staining distinguishes between them

Hill: I speak as a histologist You mentioned reticular I only wanted to get that clear for myself; it is a small point.

Gersh. Some recent analyses of tissues rich in reticular fibers, have been made — so far as amino acid goes they are very nearly like that of collagen, but there are some differences. Whether these are due to impurities to different degrees in both preparations, I do not know Reticulum fibers look different, for one thing, and behave differently with certain stains, morphologically then, the distinction may become a very useful criterion; and, as I said, chemically, it is not yet certain that the two are identical. The question of spacing is perhaps a simplification, after all.

Ragan: The precollagen described by Orekhovich (2) contains fibers with the spacing of typical collagen spacing, but there are also fibers with an axial spacing of 2200 Å (3)

Porter: That "precollagen" you refer to is reprecipitated from citrate buffer extracts of skin and has a spacing of about 2000 Å. I do not think it occurs naturally in the fibrous form I do not think anybody has reported any fiber with characteristic collagen banding with a spacing different from 640 Å

Fremont-Smith. Does not this discussion bring out two or three things right away? One, the value of having the discussion, and two, the need, if these terms are going to be used, to specify both the differences and the similarities

Mirsky: The term "collagen" has been used several times Are there not a number of collagens, rather than a single collagen?

Gersh: "Collagen fibers" is how I meant to state it That is, morphological structures which may not correspond exactly with what has been separated chemically, or analyzed chemically as collagen.

Mirsky: The term collagen has crept into general use to such a degree that a variety of conditions are now designated as collagen diseases. It should make one wonder about the danger of introducing such a term when there is much that is yet to be done in clarifying what we are talking about when we talk about "collagen"

Gersh: These are collagen fibers we have talked about

Ragan: Collagen is surprisingly similar, whatever its source, chemically or on electron microscopy

Gersh. Banga and his colleagues found differences in response of collagen from different organs

Mirsky: That is the point I had reference to.

Gersh: Collagen, in the morphological use of the term, characterized as fibrillar in structure, occurring in bundles, stainable in certain ways, and so on, may be demonstrable histologically

Mirsky I do not mind what the substance is called, so long as we are in accord as to what it is that we are talking about At the very outset we seem to have two points of view, one, that collagen is a single substance and, two, that there are a number of collagens The question that arises concerns the interpretation that will be given the term collagen by others who are not so intimately involved in the subject

Fremont-Smith: It helps immediately when you say, Dr Gersh, collagen morphologically speaking, and someone else speaks of it chemically.

Gersh: I think that could be pointed up by the difference, in acute [illegible] shows a decrease, whereas

[illegible] e ordinary staining reaction [illegible] fibrillar?

Gersh: If one talks of normal collagen, yes, if one talks of abnormal chemically there is no great difference at all

György: What would you call abnormal?

Gersh: Collagen not fibrillar, collagen which shows different staining reactions, that which shows peculiar digestibilities, or something of that sort

Gyorgy: How do you recognize it as collagen? If it does not share any of the [illegible]

Gersh: [illegible]

György [illegible]

Gersh. Morphologically, because it has a fibrillar structure which stains with aniline blue in certain ways; swells with acetic acid in certain ways, responds to pepsin and trypsin in certain ways; will be stained by other connective tissue stains, and will not be stained by other stains Chemically, it was defined by Lowry, rather specifically, in his analyses of connective tissue (4).

Fremont-Smith The question you are asking, Dr. Gyorgy, is whether abnormal collagen is collagen Whether the term is useful to you depends very much on the purpose for which you are using it.

Gyorgy You mentioned in your definition of collagen the fibrillar structure, but a minute ago you said pathological collagen has not necessarily a fibrillar structure—

Gersh: That is right, but it would have some of the other properties

Morrione The method of Lowry (4), which I think is probably the best available technique of determining collagen quantitatively, I am quite sure determines reticulum as well This is in accord with the previous statements that they are probably the same substance, except for variations in the caliber of the fiber

MacLeod. Is not one on hazardous ground in making quantitative estimations of a soft tissue component based on exposure to strong alkali and autoclaving?

Gyorgy The body basically, as far as I know, utilizes alkaline extracts of tissue first as a method of removing soluble protein The collagen then remains, together with elastin, and the selectivity of the collagen principle is the fact that it is hydrolyzable when autoclaved with distilled water, whereas elastin is not, so that in the technique, the loss of weight of collagen is what we look for

Hartroft The problem of defining and distinguishing reticulin fibers from collagen has been most perplexing to me, and at the same time of considerable import in tracing the pathogenesis of experimental dietary cirrhosis as it develops in choline-deficient rats No matter what stains are employed, including silver precipitation techniques, it is always possible to observe what appear to be transition forms between the normal reticulin fibers supporting undamaged parenchyma and the more "collagenous" material of the trabeculae The latter stain more intensely with aniline blue than do the reticulin fibers, which are better delineated by silver than are the coarser trabeculae But these differences are merely quantitative, for transition forms are easily traced If the trabeculae are derived from condensed reticulin fibers, then the question arises whether these trabeculae should be called collagenous or even fibrous as they frequently are This question assumes considerable importance when comparisons are being drawn between the experi-

mental dietary cirrhosis and that encountered in man I would like to ask Dr Gersh *if he would not agree that condensation of reticulin* fibers under pathological conditions can give rise to collagen?

Gersh I am sure that something of that sort goes on, but just when such a change takes place is very difficult to say, just as it is very difficult to say when a baby is not a baby as it gets older, or when adolescence begins and ends, or when a macrophage in transforming to a fibroblast and is not any longer a macrophage or more clearly a fibroblast. *It is something like some in-between stage which, by definition,* would be not necessarily reticular fibers or collagen

Campbell If you use the analogy of the baby, Dr Gersh, are you *suggesting that the reticulin fiber is necessarily baby collagen, or would* you allow that collagen may have a second childhood, and revert to reticulin?

Gersh I do not know

Campbell Pathologically, I think we can see strong suggestions of that

Holbrook May I suggest that you proceed with your presentation now, Dr Gersh?

Gersh The second part of this presentation concerns a definition, *this time a definition of ground substance The ground substance ap-*pears optically homogeneous with the white light microscope It varies in consistency from a gel like state to a more watery state It contains carbohydrate, and this is probably responsible for the greater part of the staining, with the method of Hotchkiss, McManus, and Lillie Under certain conditions, *it may be stained metachromatically* Normally, it is only slightly stained by Evans blue when this dye is injected intravenously as an intravital dye But under certain conditions, the ground substance may be deeply and almost selectively stained

Apart from the optical appearance four properties were mentioned consistency, stainability with *Hotchkiss* procedure, stainability with Evans blue as an intravital dye, and metachromasia We have tried to relate these *four properties in a general theory on the nature of ground* substance This theory has been used to predict successfully certain functional conditions in which there is a transformation of some or all of these properties

The theory holds that ground substance is polymerized the *term polymer being used in its widest sense to include carbohydrate-protein* complexes which are aggregated to form submicroscopic structures If one regards ground substance as highly polymerized, looking at things in this general way, then it stains weakly with a Hotchkiss procedure It is very palely stained with Evans blue, it will show little or no metachromasia, *and tends to be gel-like*

When the ground substance is considered to be partially depolymerized, it stains more strongly with the Hotchkiss procedure It will have a very marked affinity for Evans blue, and perhaps will be more metachromatic

When the ground substance is markedly depolymerized, it may stain less strongly or more strongly with the Hotchkiss procedure. It will have a very marked affinity for Evans blue It will probably be more metachromatic, and it becomes, in the end, water soluble

At this point, it may become broken down into particles small enough to enter the blood, where they are detected as an increased plasma mucoprotein level

We have then several morphological criteria for detecting what we think is the submicroscopic state of ground substance of connective tissues

MacLeod: Is Evans blue a basic dye?

Gersh: It is highly colloidal and resembles closely toluidine blue, another basic dye It is tied almost entirely to albumins and globulins in blood plasma, with very little free in the plasma as uncombined dye

Wier: I would like to ask a somewhat unrelated question In this type of experiment when you get increased staining with Evans blue, do you also get an increase in the rate of loss of dye from the blood plasma?

Gersh: The loss is very slight, and moreover, you can replace this or increase the dosage by giving additional injections in time But as it is a ten-minute period, very little loss will take place.

Gyorgy: Did I hear you correctly when you said that the ground substance escaping into the blood can be detected there?

Gersh: When it is broken down into small enough particles, then the evidence indicates that it goes into the blood, where it is detected

Gyorgy: Is that a difficult procedure?

Gresh: No, not for large volumes, on a micro level, it is rather difficult

Fremont-Smith: It is detected as mucoprotein, did you say?

Gersh: As a mucoprotein.

György: How do you detect it in blood plasma?

Gersh: By Winzler's method

Mirsky: Are we not talking about nonspecific phenomena? When you say mucoproteins entering the blood stream from ground substance, are you not speaking about a rather heterogeneous group of substances? As far as I know, even the "specific" method for mucoproteins is far from specific

Gersh: As I remember it, Winzler separated out his material in a

rather pure state electrophoretically Is that not right?

Mirsky. I believe so

Gersh: With regard to the question of the kind of mucoprotein As I said, this whole thing is a very general theory, and I do not pretend to be able to say what it is What one can do is produce situations in which two phenomena go on a) an increase in water-soluble mucopolysaccharides in the connective tissue of the region affected, and b) an increased blood level of a water-soluble mucopolysaccharide With timing and different experimental situations, it is possible to relate them not in an iron fast way, but with a high probability.

György If you inject hyaluronidase, would you get an increase?

Gersh I do not know

György: It has been claimed by Dr Butt, of Pensacola, Florida, (5) that the colloidal activity of the urine increases after subcutaneous injection of hyaluronidase

Gersh: I do not know of it

Ragan: Was the enzyme injected locally or intravenously?

Gyorgy Subcutaneously He found a high excretion of some unidentified colloid in the urine

Gersh: I will tell you some details in which this general thesis has been used, several examples in which blood analysis did enter As I say, this is general, and I do not think it is possible to make it specific.

Mirsky: I am not disputing your statement, because there is much evidence that can be utilized in support of your general thesis, but with cellular damage, and presumably the release of ground substance, there is an increase in mucoproteins as well as an increase of inhibitors

Ragan: Does an increase in capillary permeability play any role in this general picture?

Gersh: I have that as a specific section later, but I am perfectly willing to go into it now

Bennett: What are some of the conditions which you have in mind where this increase of mucoproteins occurs in the blood?

Gersh: Well, that was found, for example, where changes took place in ground substance in mice which had rapidly growing subcutaneous transplanted tumors

It took place when similar changes were induced in connective tissue in the *symphysis pubis* of guinea pigs treated with relaxin It took place with parathyroid hormone when administered in large doses It took place also in scurvy Those are the instances which I know of where similar data exist, both for connective tissue of a selected or generalized region, and the blood

MacLeod I am not clear as to how the ground substance is detected

Gersh. It is not ground substance that is detected, it is a mucoprotein

MacLeod: How has that been detected in these various pathological conditions?

Gersh. The Winzler method was used

Gyorgy. How do you relate it to ground substance? It could have been produced elsewhere

Gersh. I must repeat that there is a correlation in time of the plasma mucoprotein level with a change in the connective tissue, the change being in the direction of a production of a water-soluble material in the ground substance

Gyorgy. Did you not say that it is a breakdown product of ground substance?

Gersh: What we called a depolymerization of ground substance, the breaking up of a larger, interconnected aggregate into the smaller components which go into its formation

Gyorgy: Would it not be possible that some cells which, in turn, may produce ground substance produce also the substance detectable in plasma which never did go through the ground substance?

Gersh: If the cells produce it, then it must go through ground substance

Ragan: It is a temporal relationship If you wish to hypothesize that its source is the ground substance, that is reasonable

MacLeod: But not proven

Gersh It is not only temporal; there are specific conditions You have to do something specifically and then it happens at a certain time

Ragan: These two phenomena occur at the same time That is what I meant by a temporal relationship

Porter: Is there any possible way of detecting a change in viscosity in the tissue?

Gersh We have been talking about a change in consistency. In certain situations, as around rapidly growing tumors and the relaxed *symphysis pubis* of guinea pigs, the connective tissue is very watery and can be cut readily with a probe This is quite different from the normal connective tissue, which is sticky, tough, and rather difficult to cut.

Ragan: If one may assume that joint fluid represents a type of ground substance (and that assumption may not be valid in an inflamed joint) joint fluid hyaluronate is depolymerized during inflammation

Fremont-Smith: Other things happen to joint fluid in inflammation in addition to those you have mentioned, for example, it is diluted

Ragan But polymerization of joint fluid hyaluronate can be measured and quantitated (6)

MacLeod: Do you, at the same time, find an increase in glycoprotein in the serum?

Ragan: We have not done that

Mirsky: One may make the generalization that in any nonspecific disturbance of an organ, there may be an increase in serum glycoproteins.

I believe it has been established that the trypsin inhibitors, for example, are glycoproteins We know that they are increased in practically any disease condition in which there is some tissue breakdown Consequently, it is probable that there is an increase in glycoproteins in any state associated with marked stress For example, even an emotional response to some disturbed interpersonal reaction can cause a change in trypsin inhibitors, which, in turn, may effect a change in glycoproteins

Holbrook I think it is fair to assume that Dr Gersh believes that the rise in glycoprotein in the blood may be due to depolymerization of the ground substance Would you not all agree to that?

MacLeod: Provided one says it may or may not be due to depolymerization of the ground substance.

Fremont-Smith We have a great tendency to accept definitions as having some virtue other than the purpose for which they are made I think this whole discussion so far can be considered one of the most interesting and valuable discussions we have had I think it will make very interesting reading for other people, because these problems come up again and again

A given definition is not a definition for all time, or for all purposes It was made within a narrow context It has relatively limited virtues, uses, and it will need to be modified when one looks at the problem from a different point of view

Morrione May it not be, too, that the tissue fluid which we have forgotten about might be related to some of these changes, since it is so intimately associated with ground substance? As a matter of fact, it may be an artificial separation which we are talking about, and we

cutaneously with which we worked

When a group of cells like this increases in number and occupies a

consistency of the ground substance of connective tissue surrounding a growing part is altered, becoming more fluid, and "weaker" This was

tested on transplantable tumors, some of which grew slowly, or regressed, while others expanded rapidly.

We found that in the region of the rapidly growing tumors, the connective tissue was watery, very soft, and very fragile, so that it could be cut with a blunt probe When Evans blue was injected into the animal a few minutes before it was killed, the dye was almost selectively deposited in that same region The Hotchkiss stain, at first, became redder, more prominent . that is, at some earlier stage . . . and in a later stage there was a large water-soluble component in the ground substance

The material was collected from around the tumor, analyzed chemically by Dr Catchpole, and found to have a much higher than normal level of soluble glycoprotein of the type described by Winzler; it was also found that similarly the plasma levels were greatly increased

MacLeod. What was the nature of the tumor?

Gersh. The Earle HGW tumor composed of sarcoma cells which first appeared in tissue culture I think, and were then carried in mice

We applied the same general approach to the growth of the Graafian follicle in the ovary, where one could imagine that there could be two situations; either the cells in the Graafian follicle had to exert pressure to tear connective tissue, or the connective tissue on the outside becomes softer, the cells simply occupying the space progressively as the ground substance in the environment becomes more fluid There we found similar changes in the density of Evans blue locally, as well as in the amount of water-soluble glycoproteins, as tested for by the Hotchkiss procedure.

This general thought on ground substance and its relationship to expanding groups of cells is beginning to be applied to normal developmental processes, for example, in the developing eye of the embryonic chick At some point early in development there is an enormous acceleration in the rate of growth of the eye You all remember how, in early stages of chick embryos, the eye is the most prominent thing in the head The embryos were injected with Evans blue intravenously. Here again, when the rate of growth is greatest, Evans blue is selectively deposited in the connective tissue around the eyeball, indicating that perhaps some of the other changes I mentioned are also taking place in the ground substance of the expanding eye

Work has been done also on the pancreas and the metanephros of young chicks after hatching, at about seven to nine days for one organ, and about thirty days for the other organ, when there is a marked acceleration in their rate of growth Both with Evans blue and sections stained with the Hotchkiss procedure, the familiar changes can be made out

If this thesis were sound, one would expect that after partial hepatectomy, in regeneration of the liver, one should also find changes in basement membrane of the sinusoids, or of the liver cell cords Actual experiments showed that changes appeared in the nature of the ground substance, a kind of increased stainability, and a swelling, which corresponded in time with the more active stages of regeneration It was detectable three hours after hepatectomy, and was not detectable at about seventy-two hours, when the liver regains most of its normal weight and volume

Hartroft. What stains were employed?

Gersh: We used the Hotchkiss procedure on this, also Mallory, and H and E

We have not yet done metachromatic studies on this material, but it appears from the literature that especially at the periphery of certain neoplasms, and in certain rapidly growing organs of embryos, the connective tissue becomes more metachromatic

One might reverse the situation and propose that an organ stops growing at least in part because the connective tissue becomes denser, or more rigid, imposing a greater obstacle to the spread or multiplication of cells It is possible that this local factor is involved in regulating the growth and perhaps the form of organs during normal development

One could think of this increased polymerization of ground substance, as we think of it, as an aging process I should like to mention now some observations on the aging of ground substance

Evans blue is distributed in the ovary in the following manner it is deposited in the connective tissue, in the *zona pellucida*, and in the lumen of the Graafian follicle, where some reactive material is present I think some can be detected in the plasma of the blood vessels It is a very sharp reaction

In a diagram, showing some parts of liver cell cords in a normal animal, the unstained nuclei are outlined The particles in the cytoplasm of the sinusoidal cells are of different sizes, density and color Six hours after regeneration, the morphology changes The basement membrane, which in the normal is just barely noticeable, becomes more prominent in spots, and frequently it is thicker A little later, it becomes markedly colored by comparison with both the six-hour and the twenty-four-hour stage For the most part, the material in the sinusoidal cells is distributed as granules which are of more uniform size, and stain more or less similarly

In a normal liver, only in spots does one see a very thin line The nucleus is unstained and indicated as a clear region In an early stage

of regeneration, it is possible to see a basement membrane in some places It is a very thin, faintly stained line A few hours after regeneration, one can see a sharper basement membrane, and a thicker one The cells lining the sinusoids have many more granules of this reactive material, and they are very nearly of the same size and frequently of the same tint.

Bennett: When you speak of reactive material, are you referring to Hotchkiss-positive material?

Gersh: Yes

Hartroft May I ask if you feel that this change is sufficiently specific that it might be used generally as an index of hepatic regeneration?

Gersh: Not for humans, where one does not know the previous condition, or where one could not even assume the normal state of the basement membrane existing at any selected part which might be taken for study as normal

Hartroft I am thinking more specifically of livers in experimental cirrhosis.

Gersh I think it could be A much better indication, as it turns out, is the presence of these granules in the sinusoidal cells, which can be seen with the low power of the microscope, in contrast with the basement membranes where one must use Kohler illumination

In the skin of the rat just before birth, most of the staining in the epithelium is due to glycogen The basement membrane around the capillaries is barely perceptible It becomes prominent both under the epithelium, and around the capillaries just two days later, and becomes thicker in young animals. and markedly thicker around blood vessels and under the epithelium in very old animals.

In the thyroid, normally a very thin basement membrane is detectable around the epithelium of the acinus The colloid is stained deeply, but the basement membrane in the normal is very thin It is interesting that in a number of young hypophysectomized rats the thickened basement membrane to all intents and purposes duplicated the appearance of that in very old normal rats, normal in the sense that there was no history of any abnormal events

Talbott: These were young, not mature rats?

Gersh These were young but mature rats They were about the same weight and about the same age, and the period after hypophysectomy was only about two weeks

Shortly after the administration of an extract of the anterior lobe of the pituitary gland containing thyrotrophic hormone, black droplets appear in the cytoplasm

Hartroft. Are the changes in the hypophysectomized animal specific

only for the thyroid, or are they found throughout other organs generally, particularly, the islets of the pancreas?

Gersh: A similar effect took place in the testis. I have not yet looked at other material.

This completes what I have to say on growth and the possible role of ground substance in growth. Before we begin the discussion, I should like to point out that I have other material to discuss, in relation to various endocrinological reactions, capillary structure and possibly permeability, anaphylaxis, and also to calcification.

MacLeod: As I recall, at the Conference last year there was discussion about the specificity of the Hotchkiss reaction, and I wonder whether in the interim that has been resolved, that is to say, whether the reaction is specific for glycoprotein.

Gersh: I could state my attitude, if you like, having read the prior discussion. I think it is clear that one can stain glycogen. On the other hand, one would not stain compounds like glucose, or other water-soluble substances of that nature.

In frozen dried material, it is possible to remove, so far as we know all the lipids, a number of which do react with this procedure, by the use of hot chloroform-methanol extraction, for a long period of time. There remains then the complication of possible reaction with certain amino acids, and the staining of carbohydrate combined with protein in some form.

Which of the polysaccharides combined with proteins are stained cannot be ascertained by this method. It is not possible to say that a polysaccharide is sulphate-containing, or that it is composed of a glucosamine, or a galactose compound or hyaluronic acid. Compounds which have reactive groups exposed are stained. Treatment with water, with acid, or alkali, will expose a greater number of them, and a deeper stain under certain conditions is developed. I think, from our point of view, it has been extremely useful to be able to characterize the stainable material as water soluble, or water insoluble. It is not with certainty specific for glycoproteins, but the probability is that most of the material that is stained in ground substance is glycoprotein.

Mirsky: Is it the branching that determines the staining?

Gersh: It would seem to be the degree to which the reactive groups are covered up by the close packing or degree of polymerization.

Bennett: In association with this increased thickness and stainability of the so-called basement membrane, which you have just talked about, are there visible changes in other components other than the reactive material? More specifically, is there a change in the reticulin fibrils, or of the collagen fibrils, within that structure, or is this phenomenon due

entirely to an increased prominence caused by a reaction with the Hotchkiss-M[illegible]

Gersh: I [illegible]

distributed [illegible]

are changes in the sinusoidal cells, as you have noted and in the liver cells Other changes can be detected

Dempsey: This means that in your preparations the reticulum does not stain?

Gersh: It is not recognizable as such We see a homogeneous material which in a manner of speaking we correct for reticulum which might be in it

In only one situation, as prepared by freezing and drying, have I ever seen a very fine stain of reticular fibers, and that was in the spleen of a patient with Gaucher's disease, where the reticular fibers were beautifully and almost selectively stained

Dempsey It is hard for me to differentiate what you showed in the liver from reticulum There were basement membranes which were visible as very fine lines, just about the dimension of the reticulum fibers which are known to be in that location How to differentiate the visible object that one sees—

Gersh You could see reticular fibers by other procedures with the application of silver stains, and these were different. Where we could not see, or could barely see, a basement membrane, reticular fibers were quite numerous

That does not cover another situation, where the material may be water soluble, and much of the reticular fibrillar material would remain behind, with very little staining The Hotchkiss method may be used to stain many structures It can be used in a situation where it may be profitable without becoming involved in complicated situations which are not analyzable

Dempsey: I am still interested in this matter of the reticulum, and how to differentiate it from the homogeneous ground material, since reticulum does stain intensely with the periodic acid-Schiff reaction, at least in chemically fixed material

Gersh: It does not after freezing and drying.

Dempsey Insofar as our chemical knowledge goes, the reticulum should have groups which react with periodic acid, I do not understand why it does not stain after freezing-drying

Gersh In many preparations, one sees holes, as if they were composed of some gas but which are artefacts Things were there before which had been removed, or had shifted from that spot and moved to another spot I think that is why one sees reticular fiber staining in material

Dempsey You propose, then, that the reticular fiber in chemically fixed material stains because the ground substance has precipitated upon it?

Gersh Has shifted toward it, yes

Dempsey So that after ordinary fixation, you believe that one actually stains the same material which you are staining in frozen dried material However, after chemical fixatives, you believe it has shifted its location and has been adsorbed upon the fibers?

Gersh I would not say "the same," but I would say something of the same sort

I should like to discuss the role of ground substance in certain organs having normal cyclic endocrine processes

I will abbreviate the factual material by summarizing briefly the work done by Miss Perl and Dr. Catchpole on the *symphysis pubis* of guinea pigs (7), where there is extensive softening and resorption of both bone and connective tissue over a short period of time

The early stages of this process can be produced by the administration of a plasma fraction containing relaxin after the animal had been primed with estradiol In the normal animal treated with Evans blue there is very little blue present in the connective tissue around the symphysis In the animal treated with estradiol, some [illegible] s deposited In the animal treated with [illegible] able concentration of Evans blue is [illegible] symphysial connective tissue In t[illegible] animal, with a wide symphysis, much Evans blue is present

This was correlated with a change in the solubility and stainability of the ground substance and recently, others have found that the connective tissue from this region has an increased concentration of water-soluble protein

I have already talked about ovarian changes (8), with emphasis on the growth of the Graafian follicle Those changes were fine enough to be seen in the normal estrous cycle, and became greatly exaggerated with the administration of gonadotrophin

Angerine Is this ligament composed of dense connective tissue?

Gersh The parts that are stained by Evans blue are not primarily the ligament, but rather the pre- and postsymphysial connective tissue

Angerine What type of material is it?

Gersh The material in the *symphysis pubis* is microscopic in size, one pubic bone cannot be budged from the other readily It is very dense On the other hand, the administration of relaxin results in some degree of movement. However, the greatest change was found in the connective tissue on each side of or around the *symphysis pubis* itself

Gyorgy: What kind of change? Is that new connective tissue, or old transformed?

Gersh: If one considers connective tissue not as static, but as being continually removed, renewed, and replaced in a normal individual, the question of new or old connective tissue does not arise.

It is possible that normally the ground substance is also changing, but that the rate of change is altered under certain conditions.

It is clear from endocrine studies that the connective tissue of endocrine glands, particularly the ground substance, undergoes changes concomitantly with those of parenchymatous structures. It is possible that alterations in the physical state of organization of the ground substance may be a necessary activity without which the well-known cellular changes in endocrine organs may not be possible Hormone action on a target organ may then be on two sites, the connective tissue cells, whose response affects connective tissue components, and the parenchymatous cells

At any rate, our findings indicate that hormones which act on connective tissue cells have a high degree of selectivity, or specificity, i e, relaxin and the Graafian follicle. It is as if connective tissue cells which are indistinguishable morphologically in different sites belong to different families, each of which differs from its neighbors in sensitivity to specific stimuli This is not a new concept — it has been invoked to explain certain instances of ectopic bone formation and the mechanism of action of growth hormone

There may be other instances of specialization of connective tissue cells, and it has been proposed that certain phenomena of menstruation, placentation, growth of the mammary gland, exophthalmos, and the rapidly proliferating uterus, represent extensions of this general concept that connective tissue cells show a certain kind of specificity in different sites, though morphologically they might be alike

We move next to a consideration of the preference which metastases of certain malignant tumors show for enlargment, survival, and growth If one should regard as similar the offshoots from malignant growths into the vascular or lymphatic system and gas or fat emboli, then one should expect that the cellular emboli should be distributed throughout the body, depending on the rate and volume of blood flow, the nature and dimensions of the vascular pattern, and similar factors. Yet offshoots of certain carcinomas give rise to metastases which lodge in preferential sites, which on the surface, appear bizarre Our explanation of this phenomenon runs as follows. Let us suppose that the tumor-emboli have a vascular distribution essentially similar to that of emboli of other carcinomas, or of fat or gas emboli They lodge in numerous sites and die or remain quiescent in most sites with an unfavorable

extracellular environment But in the one or few bizarre sites, the emboli cause the local connective tissue cells to alter the ground substance, so that the environment becomes more favorable for survival, growth, and spread of the metastases

This suggested mechanism depends *then on a fundamental difference* in sensitivity of certain connective tissue cells differing in degree from *those of all other connective tissue cells*

We turned our attention from there to what I think is the most specific change we found The experiments were done with Dr Rappaport at the University of Illinois on guinea pigs and rabbits sensitized to horse serum, and then injected with Evans blue We used the site of deposition of excess Evans blue as an indication of the site in which a change in consistency of ground substance might have taken place

In the lungs of anaphylactic guinea pigs, the deposition of dye was particularly marked in the connective tissue surrounding the large bronchi and some of their branches, while the ground substance in adjacent regions was barely stained with Evans blue (9)

Now, by contrast, in rabbits similarly treated there was a marked excess of dye in the *tunica muscularis* of the large pulmonary vessels

Gyorgy What happens in the animals after you elicit the anaphylactic shock?

Gersh. The guinea pigs all died

Gyorgy What happened with the stains? Would they stain the same after the shock?

Gersh The animals were injected with Evans blue five to ten minutes before they were challenged, so that they were already prestained *Presumably if the animals had not been shocked*, as we found in our controls, there would be very little stain in these specific sites There was enough to see *a selective deposition in one region in one animal*, and in another region in another animal It is interesting that the people working in anaphylaxis have, by indirect procedures, come to the conclusion that the very same regions are affected in these different animals

Ragan These same regions show an increase in capillary permeability during anaphylaxis

Gersh I do *not* know exactly what you mean by an increase in capillary permeability

Ragan Substances leave the *capillary* bed which *under ordinary* circumstances do not

Gersh Just *perceptible, very little But in the animals in shock there* was enough dye to see that it had been selectively deposited in one region in one species, and in another region in another species

Wier. Were your changes in scurvy localized in a similar fashion, or were they more general?

Gersh The findings in scurvy were somewhat different There was, you might say, a return to a fetal state in that the ground substance was readily water sol [illegible]
also as in the fetal s [illegible]
nective tissue quite [illegible]
creased number of these granules that we call secretory granules, these glycoprotein-reacting granules Dr. Perroni and Dr. Catchpole have found that the blood mucopolysaccharide level is high It has been reported in the literature that the collagen chemically determined in scurvy does not differ greatly from the normal. In such a situation ground substance is being formed continually by connective tissue cells, and is passing into the circulation Given suitable conditions, that material, and probably all the newly-formed material, would revert toward a more highly polymerized state

Gyorgy How is the mucoprotein content of blood during anaphylactic shock?

Gersh I do not know.

Campbell What effect has local administration of histamine, Dr Gersh?

Gersh We did only a small number of rabbits with histamine. The changes were similar to those treated with protein when these animals showed similar symptoms

Ragan Have you done anything with leukotaxine?

Gersh No

Ragan There is a question about whether or not there is a turnover of any speed, in connective tissue protein Sprinson and Rittenberg (10) have done some work on carcass protein, and in the rat its turnover is very slow, compared to the water-soluble proteins in liver and in circulating serum proteins

Gersh I do not know exactly how the experiments were done, but I could present a possibility in which such a condition might be so If one considers an aggregate at one site, and one part of it is chopped off and becomes smaller, it may be captured at another site, or it may go into the blood, and become incorporated locally at another site Then some part there would break up and shove off somewhere else. In that situation, you do not have to have very much of a metabolic shift.

Ragan The work was done with N15 in the rat, and the half-life of the carcass protein was twenty-one days The half-life of serum proteins in the rat is much less than that. By analogy in man, most of the protein turnover could be accounted for by serum proteins However,

I suppose you can say that the proteins are in a dynamic state and are going in and out of the tissues and into the serum all the time

Gersh: Now, as rate has come into the picture, I should like to point out that some of the experimental findings take place with surprising rapidity In anaphylaxis the changes were noted in minutes Marked changes in bone took place, or were found in six hours, and might have occurred earlier Changes in the ground substance of the liver took place in three hours after partial hepatectomy Changes in the *symphysis pubis* were found in six hours, and could have occurred much earlier

Holbrook. Is it your concept, Dr Gersh, that with Evans blue you are measuring the depolymerization of ground substance, and thereby locating the particular area of connective tissue that may be affected in a given condition?

Gersh That is right

Hill In anaphylactic shock with Evans blue, was the middle coat of the large arteries of the lung affected?

Gersh In the rabbit

Hill If I may indulge in idle speculation, I wonder if that is because it is the site of the *vasa vasorum* in that muscle We know that the reaction in the different media may differ

Apropos of that, it also strikes me that in syphilis this is the site in the larger vessels of the location of spirochetes I wonder if there is any relation between the change of the connective tissue and the lodging of the spirochete

Gersh It did not take place in the guinea pig where the change was in a different place—around the bronchioles

Ragan Were there any changes in the rabbits' kidneys, Dr Gersh?

Gersh I did not look at them

Mirsky If I understand you correctly, you are proposing that different areas in the organism with similar cellular structure respond differently to a humoral agent How have you eliminated the possibility that the different parenchymal cells are responding to the hormone, and in turn affecting the ground substance?

Gersh I did not say I eliminated that, you know, I was merely proposing an alternative which seems more promising and simpler In the *symphysis pubis* there is no parenchyma, so to speak But it is impossible to deny, from our work, that the effect might be primarily on the *parenchymatous cells, and that the local connective tissue cells have* nothing to do with it

Fremont-Smith But they respond to the change

Gersh That is right It seemed simpler, largely because of the relaxin experiment, where pathological changes take place in connective

tissue unrelated to epithelial structures and also because of the work on the developing eye

Fremont-Smith: There is something that perhaps ties in here. I can remember that the reaction of the stroma has been emphasized again and again in trying to make the diagnosis of carcinoma, or of malignancy. In metastases, you have a connective tissue stroma response which supports the blood vessels, thereby making it possible for malignant cells to grow.

Gersh: We had thought of that as probably being the basis for attention to connective tissue in the diagnosis of sections for malignancy. One would expect that there would be a softening of the connective tissue, and a breakdown and possibly solution of the ground substance. Then after the usual fixation, little ground substance would be detectable

Fremont-Smith: Is the change in the stroma of the metastasis specifically positioned there, as well as around the periphery of the metastasis, which is apparently being prepared for the metastasis?

Gersh: The same change takes place but in the connective tissue stroma, continuous with the unorganized capsule of the transplanted tumor.

Mirsky: Since we are permitted to fantasy all we wish here, how would you explain your concept of specificity of responses in different areas of the body to humoral agents, when, so far as your description has gone, we are dealing with similar structures and similar components? There has to be a difference to begin with

Gersh: When one looks through the microscope at sections of glands, it is difficult for one to know whether that gland has been secreting actively just before it was removed and fixed, or whether it was just "resting" without putting out much material. This is particularly true of mucous secreting glands. Something excessive is needed to show up differences in activity. Similarly in connective tissues, though the cells may look alike it is possible that their composition, their cytogenic disposition, or sensitivity may be different

Mirsky: Then the inference would be that actually we may be dealing with structures in the connective tissue in different areas sufficiently different so that agent "A" will produce an effect in only one area of the connective tissue and not in another, and sufficiently different so that the reactivity, or the responsiveness, is not the same

Gersh. Yes

Mirsky: This concept may be important. That idea could be put to test in time, and if such an inference is established, it opens up a large field which may lead to the understanding of the different responses to nonspecific agents

György I do not think it is necessary to assume different chemical structures Would it not be possible to assume you have first a hormone, like relaxin? Relaxin has, in the target organ, some substance with which it will interact Through the interaction at this local place, the connective tissue, which has the same chemical composition in the body, will react.

Mirsky You would have to have something different in the symphysis than you have in the liver

György The connective tissue is the same

Fremont-Smith You have gone back to the alternative proposal of the hormone acting primarily on the parenchymatous cell, which is not the proposal of Dr Gersh

Mirsky There are two possibilities Either it acts on the parenchymatous tissues which change, producing something that alters the medium for the connective tissue, or the action is directly on the connective tissues

Travell What would you regard as the parenchymatous cells in the symphysis pubis?

Gersh They are connective tissue cells

Travell Then there are no parenchymatous cells of the type that Dr Mirsky is talking about

Mirsky. Growth hormone affects the epiphyseal end of bone to a greater degree than it does other structures

Gersh That was cited, also the ectopic formation of bone Huggins (11) was unable to induce bone growth anywhere, except along the midline of the anterior abdominal wall

Mirsky The studies of Trotter, concerning the question of why metastases cannot occur in the central nervous system bear a direct relationship to what you have proposed (12)

Angevine What do you mean "cannot occur"?

Mirsky He made quite an issue about certain types of metastases not occurring in the central nervous system

Hill What is your opinion about the role of the fibroblast? Does it produce this ground substance we are talking about, or does it secrete an enzyme which acts upon it, or does it act as a reinforcement?

Gersh Being simple minded and not having access to sources of knowledge which are not available to anyone else, I would say that the connective tissue cells, not necessarily the fibroblasts, may produce the ground substance and are also involved in controlling its state of polymerization Thus the same cell might make, by *remote control*, a highly polymerized material and might also break it down

There are instances in histology of cells which, let us say, at one

know Ground substance is probably secreted by the fibroblast.

Porter. Does it have to change to a macrophage to do that?

Gersh: Not so far as I can see

Hill: May I discuss a few figures which I think are pertinent to the discussion? In the West Indies, we have observed a liver disease in which the peak incidence takes place in children up to four years old Our youngest case is four months, and the only etiological agent we can find is a low protein diet.

In 25 per cent of our cases, there is a serous exudation between the sinusoids and the liver cell columns, also around the central lobular vein Figure 1 shows what we call a serous exudation

Figure 2 also shows serous exudation in another case, that of a child about two years old, there is a deposition of eosinophilic coagulum In Figure 3 can again be seen a serous exudation with a deposition of coagulum This is the third episode showing the most recent



FIGURE 3 Liver of child aged 1 year 3 months showing (a) collagen cuffing of centrilobular vein from previous attack, and (b) serous exudation from current attack Modified Mallory connective tissue stain × 150

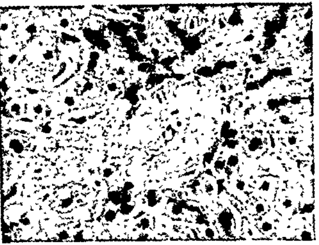


FIGURE 4 Shows waxy like eosinophilic coagulum in the perisinusoidal space The coagulum gives all the tinctorial reactions of collagen H & E × 600

disturbance around the centrilobular vein where exudation appears as an acellular fibrillar, coagulum

Figure 4 stained with H & E shows the sinusoid, the flattened liver cell columns, and in the perisinusoidal space an eosinophilic coagulum, which looks like wax, and is not metachromatic

Figure 5 is a section taken near Figure 4 The endothelial lining of the sinusoid is well shown The coagulum stains as blue blobs The coagulum is not dissolved by trypsin, contains no cells or hemoglobin, is not fibrin, tinctorially, but is an eosinophilic coagulum giving the tinctorial reactions of collagen

Figure 6 is another section taken near Figure 4 and is stained for reticulin The reticulin fibers are broken and swollen and stain black, stuck to them are blobs of yellow-brown substance which is the coagulum Perhaps collagen was formed by the addition of new amino acids, in the form of blobs, to these reticulin fibers already present With Mallory's phosphotungstic acid stain the coagulum is reddish-brown, the same as collagen, not blue All of the perisinusoidal fibers throughout the lobule have little reddish globules on them We thought this was an artefact but in livers from England they were not present, so it may be something new

We have not yet solved whether there is a proliferation of reticulin fibers, or whether there is a swelling of reticulin fibers in the ground

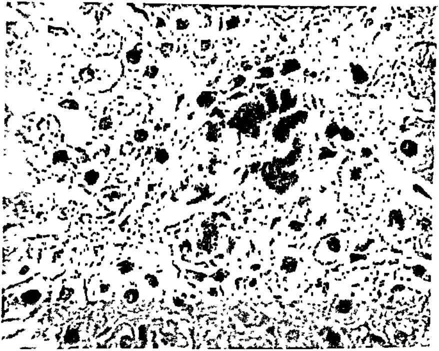


FIGURE 5 Serial section of Figure 4, 16μ deeper and stained with Mallory's connective tissue stain Note the blue staining coagulum in the perisinusoidal space the sinusoid is clearly seen $\times$ 600

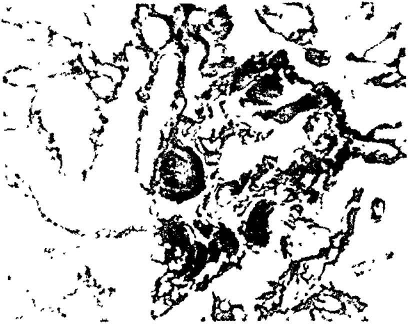


FIGURE 6 Serial section of Figure 5, 8μ deeper and stained with a modified Foot-Wilder reticulin stain the reticulin fibers are swollen and stain black but the "blobs" of coagulum are golden yellow in color × 600

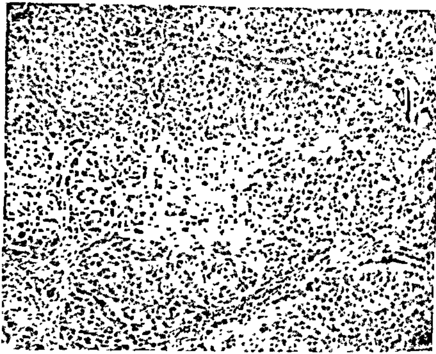


FIGURE 7. Shows wide-spread deposition of eosinophilic coagulum throughout the lobule in a liver of a child aged 3 years 3 months H & E × 150

substance, to which protein from the serous exudation becomes adherent

When I went out to the West Indies eighteen months ago, I complained to my technician, an excellent craftsman, that the slides were badly stained, and that there was something wrong with the collagen staining He said, "The collagen in Jamaica stains differently from that in England"

These children with liver disease are on a low protein diet, and develop "collagenosis" in their livers. After that, the fibroblast appears with subsequent fibrosis It was as if concrete or collagen protein were poured in, and later reinforced by iron bars—the fibroblasts This is, of course, theory

Figure 7 shows the typical lobe showing "collagenosis." There are a few cells, I will admit, since this has been going on for some time It does not give the stains or reactions of hyalinosis, or scar tissue.



FIGURE 8 Liver of a child aged two years showing fibrillary collagen from previous attack and serous exudate from current attack Modified Mallory connective tissue ×600

Figure 8 shows a case with repeated attacks There is an eosinophilic coagulum around a vessel This stained with Mallory's stain, has a fibrillar structure, but as yet no fibroblasts have appeared It is possible that the fibrillar arrangement is due to stress, we do not know

Figure 9 is a section of liver from a child four months old, glycogen is heavily stained in the cells with the Hotchkiss stain There is a faintly staining band of wavy fibrils in the space of Dissé, —the perisinusoidal space—with no cells This band of wavy collagen extends from the portal triad into the lobule

Subsequently fibroblasts will invade and fibrosis will develop

Holbrook I would like to ask Dr *Hill* two questions on the material he has presented His impression was that these liver lesions were related to a particular type of low protein intake, but not seen in other instances of protein deficiency First, I wonder whether he has found this unusual collagen in the livers of the population at large, who do not appear to have this particular liver disease Second, why may this

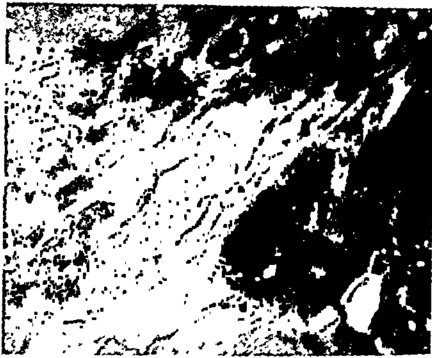


FIGURE 9. Shows a fibrillary band of eosinophilic coagulum extending from a portal triad (lower left hand corner) into the lobule diag-

condition not be due to the result of infection, or some other extraneous factor, instead of low protein intake?

Hill We now examine the liver in all cases For instance, a case of eclampsia showed this "collagenosis" in the liver A boy who was ill from some other cause—it was not liver enlargement—showed the same sort of thing.

The economic circumstances in Jamaica are very poor, and we think this liver condition comes from one economic class, where, for example, a woman with four children lives on a dollar a week. There is a very low protein intake throughout the whole of Jamaica.

Fremont-Smith: What is the staple diet?

Hill: For these children, the pattern is white bread, corn meal, green bananas The actual protein intake of a child who is well fed in Jamaica contains 35 gm of protein a day; our liver case will have 10 gm. of protein which is much below the standard—there is also little animal

protein In ten cases I can think of, seven had condensed milk as the only source of animal protein

Holbrook: You think it does occur in the general population to some extent?

Hill: I think so With regard to infection, our investigation is, at this moment, somewhat incomplete However, in regard to parasites, out of 120 cases surveyed only four or five had parasites Kahns were positive only in three cases, sickle cells in one or two We cannot find anything in the way of infection or parasites to explain this It does run in families and we have kept infective hepatitis in mind, but histologically this is not the picture of infective hepatitis

Holbrook: Have you been able to produce any similar type of "collagenosis" experimentally in animals with any given degree of protein starvation?

Hill: No, we have not done that. This has been described, more or less, before, by Rossle (13) as serous hepatitis

MacLeod The disease described by the Gillmans was ascribed mainly to a low protein diet too, was it not?

Hill Yes. When I first saw liver disease in Jamaica, of course, I immediately thought of "infantile pellagra," the type of thing described by Cecily Williams (14) or the Gillman brothers (15) and Altmann (16), as *Kwashiorkor, in which fatty metamorphosis is* present in the liver In one hundred and twenty cases, we have had only *five cases with fatty metamorphosis, and despite this,* "collagenosis" was present

Angevine What is the status of cirrhosis in Jamaica? Is it any different than anywhere else?

Hill There are really no worth-while vital statistics Waterlow (17) in his survey five years ago estimated that 10 per cent of the children he examined had enlarged livers

Campbell There is one point I would like to pursue a little further You suggested, or you quoted your technician as claiming, that collagen did not stain as collagen in the West Indies Does that apply only to the liver lesion? Do you find any evidence, in these children, or in the population in general, of atypical collagenosis in other organs?

For instance, what about the pancreas in these children, apropos of the Uganda work, where pancreatic fibrosis was part of the picture, according to Davies (18)? Did you find any evidence of collagenosis there?

Hill The Johannesburg work had hemochromatosis in that series, did it not?

Campbell Yes

Hill. Regarding my technician's statement, it was an over-all statement referring to the whole body made a year and a half ago. I am only directing my attention to it now.

Campbell. You do not see anything clear cut, though, in the form of collagen disturbance in other organs?

Hill: Of course, we have postmortems, which unfortunately at this time we have not worked up. I have had a look at the pancreas; and only in one was there a fibrosis

I would like to have the opinion of the group as to whether or not what I showed you is collagen, because I think it is, tinctorially, at least

Angevine: It seems that the first material you showed is fibrinoid The smaller surrounding fibrils certainly have the appearance of collagen

Hill: With regard to fibrinoid, this material does not give the tinctorial reactions of fibrinoid at all; it gives the tinctorial reactions of collagen

Angevine· I mean in the very earliest lesion, when it just showed around the vascular spaces Later, it looked a little like collagen.

Hill When do you call it collagen? That was why I was questioning Dr Gersh this morning Is the material in Figure 6 actually collagen at that stage, or precollagen, or collagen protein, or what have you? The fact that it was not fibrillar, which it does become eventually, does not rule out that it was not collagen protein

Fremont-Smith· Have you tried the fibrinoid stain on the earliest lesion?

Hill: No With regard to the reticulin fibers, we have not yet decided whether there is a proliferation of these, or whether there is just a swelling

Angevine· Several people have mentioned silver staining This probably is not a true stain at all, merely a precipitation of metallic ions on a fiber The other stains we are talking about really become incorporated chemically with the substance stained When silver is precipitated on the surface of a material, it is difficult to say whether there is chemical affinity or not

Hill. There are two points that occur. Is this material derived from swelling of the ground substance, or is it poured out from the bloodstream?

Porter You said it was acellular and that you saw no fibroblasts in it There may have been some there before you looked at it

Hill· But a fibroblast does not usually come and go In my opinion as a histologist, there are no fibroblasts.

Porter· The fibers are there before fibroblasts come in.

Hill: What fibers?

Porter What you are calling collagen fibers

Hill This is a coagulum Sometimes you can see acellular fibers, but I think that is a later stage

Morrione About fifty years ago, there was talk of serous hepatitis, and I believe it was Eppinger who thought that this transudate was transformed directly into collagen We have snickered at that since, and now we are back to it again

Ragan You are back about thirty-five years, too, when Bartsell (19) was talking about collagen being laid down without fibroblasts being present, and then Stearns (20) demonstrated that the fibroblast had to be present before collagen was laid down

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CHEMICAL MORPHOLOGY OF ELASTIC FIBERS

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I THINK MY discussion will tend to amplify the trend that developed earlier, to the effect that elements appearing similar in some respects within the body may actually vary either morphologically or chemically

In regard to elastic tissue, the data that I will present will indicate that elastic fibers throughout the body are not the same morphologically nor the same chemically They do not stain the same way, although there may be a few common denominators throughout these various classes of elastic fibers

May I add, at this time, that the original data that I will describe are a product of my efforts and those of Dr Rosenthal, Dr Dempsey, and Dr Alex of our laboratory If I make any sage comments, any sound remarks, they are a product of this group, if I say anything foolish, it is Lansing only

I should like, if I may, to turn the clock back to simple morphology, to review the anatomy of some of the elastic components illustrated by the following figures.

Figure 10 shows a section of *ligamentum nuchae* from a horse This section is at 380 diameters and has been stained by the Verhoeff technique I use this stain because it lends itself well to photography It is an iron-chloride-hematoxylin procedure, with iodine differentiation giving a blue black elastic fiber One obtains the same result with orcein and resorcin, but this is good for photography

A dense mat of uniform elastic fibers which make up *ligamentum nuchae* can be seen There is one that is fairly well isolated These fibers anastomose very freely Here and there, one can get the suggestion of a branch These fibers, of course, have no specific length; they branch on and on, as do cardiac muscle fibers However, the diameter of the *ligamentum nuchae* elastic fiber is quite constant, and without attempting precision, these are between 4 and 5 micra in diameter They are characteristically thick heavy fibers

Figure 11 is a Verhoeff stain of loose connective tissue of a rat, and here again the elastic fibers are very dark and branch freely The diameters are somewhat variable, but note now the difference in the char-



FIGURE 10 *Ligamentum nuchae* of horse. Verhoeff elastic stain, × 380.

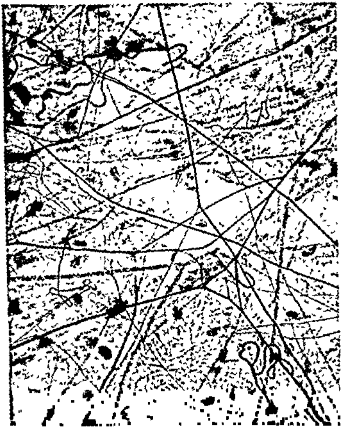


FIGURE 11. Loose connective tissue spread of rat. Verhoeff stain, × 380

acter of the fibers They are roughly 1 micron in diameter, whereas the previous were 4 to 5 micra As a matter of fact, some are even less than 1 micron in diameter Morphologically, they are not comparable to the previous fibers that we saw

Figure 12 is taken from the foot sole of an elderly individual. This shows the distribution of elastic fibers deep in the dermis There appear to be two classes of elastic fibers present; some which are rather coarse and branching, somewhat like the elastic fibers that we saw in the loose connective tissue spread, and also a dense network of exceedingly fine fibrils, which range down to the limit of visibility, probably in the neighborhood of 0 2 micron in diameter. These are very short, branch freely, tend to form clumps, and sometimes are even granular in character

As mentioned a little earlier, the heavier fibers are in some respects similar to the elastic fibers of the loose connective tissue spread. The fine fibers represent another morphologic variant of the elastic fiber pattern.



[illegible] stain, × 200

In Figure 13 is illustrated a limited field of the media of an aorta. This is a human aorta, which, as you can see, is damaged through freezing of the tissue. There are a number of artefacts throughout this section but the elastic tissue is well preserved. The elastic elements exist as dense plates clearly recognizable in a longitudinal section which we ordinarily think of as discrete elastic fibers.

Actually, as you will see in Figure 14, which is a frontal section—a section cut parallel to the lumen of the vessel—we are dealing with a dense mat of elastic fibers of different dimensions, varying from [illegible] 3 micra down to the limit of visibility [illegible] holes and fenestrations that [illegible] Figure 15 will give a fairly a[illegible] of the architecture of the elastic lamellae that ordinarily are incorrectly referred to as elastic fibers.

This is taken from Dees' paper in 1923; illustrating at least two and possibly three elements in the elastic lamellae. First, there is a coarse



FIGURE 13. Media of human aorta. Verhoeff stain, × 380.

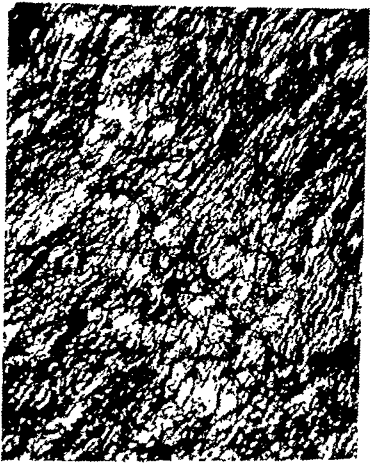


FIGURE 14 Frontal section of same block of aorta as in Figure 13 Verhoeff *stain*, × 380

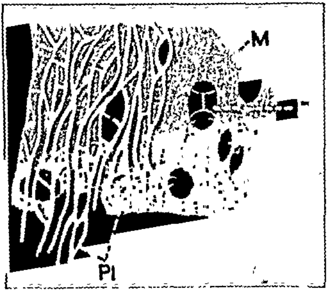


FIGURE 15 Architecture of elastic lamellae *F*, fenestra, *Pl*, coarser plexus of elastic fibers spread and adherent upon *M*, the fenestrated membrane proper Reprinted by permission from Dees, M B *Anat Rec 26*, 169 (1923)

network of elastic fibers similar to that illustrated in the preceding section of human aorta It is a very coarse network of branching elastic fibers, *not unlike those of skin, or even ligamentum nuchae* of a reduced diameter.

Next there is a very fine network of fine fibrils, which take the elastic stain These fibrils at least in their dimensions are very different from those in the coarse network According to Dees, the coarse network is apparently on one side of the elastic lamellae, that proximal to the intima In all the preparations I have seen thus far, that has been the case

So we have the coarse elastic fibers, the fine elastic fibers, and we also have a *peculiar material that I think is best characterized as a* matrix material upon which these fibers rest The elastic lamellae may actually contain three elements, rather than one, certainly at least two, if one combines the thick and thin fibers into one

These morphologic variants of elastic material do not stain similarly There are a number of significant tinctorial variations which I will summarize briefly (Table 1) The horse *ligamentum nuchae*, the arte-

TABLE I

Summary of Some Staining Characteristics of Elastic Tissues

Stain	Ligamentum Nuchae	Arterial Elastic Tissue	Loose Connective Tissue	Dermal Elastic Tissue (Human skin)
Verhoeff	+	++	++	++
Resorcin-fuchsin	+	++	+	+
Orcein	+	++	+	+ but variable
Nile blue sulphate	++	+	*	+
Congo red	red	young is red, old is yellow	red	mixed red and yellow fibers
Schiff reagent	—	—	—	—
Periodic acid and Schiff reagent	+ weak	+ in periphery of the lamellae	+ weak	+
Mineral content after micro-incineration	—	— young ++ old	*	+ variable
Osmic acid	+ weak	+ weak	*	+ weak
Victoria blue		young is dense blue, old is faint blue	*	+
Sudan black	+ weak	+ weak	*	+
Sudan IV	—	—	*	+

\+ = positive reaction
\++ = strong positive reaction
* = not tested
— = negative reaction

rial elastic lamellae, the rat connective tissue spread, and the elastic fibers of human skin, give the same general staining characteristics with the Verhoeff procedure

Using resorcin-fuchsin, we find that ligamentum nuchae stains much more lightly than connective tissue spreads or horse skin under comparable conditions.

Arterial elastic tissue has a marked affinity for resorcin-fuchsin It gives a dense, two-plus reaction Similarly, orcein will stain *ligamentum nuchae*, but rather weakly Elastic fibers of connective tissue or skin take it, but again, in skin, some fibers take the dye deeply, others take it lightly In the elastic elements of arteries, elastic or muscular, there generally is a pronounced affinity for orcein

If one turns to the mineral content of elastic tissue, using microincineration to characterize the presence or absence of mineral, we find that, by and large, there is no mineral in *ligamentum nuchae* elastic fibers

I have not examined incinerated connective tissue Horse skin does contain variable amounts of white ash that we associate with calcium and/or magnesium Arterial elastin may contain huge amounts of calcium

If one applies a leucofuchsin reagent, the so-called Schiff reagent, to elastic fibers, there is uniform lack of staining of elastic fibers when the material is formalin fixed

Now, I do not wish to imply at this time that that is a general phenomenon There may be cases in which formalin fixed elastic tissue may give a positive coloration with leucofuchsin reagent In the series I have studied, formalin fixed material is negative when the Schiff reagent is applied

If the elastic fibers are oxidized, either with periodic acid or other oxidants, one then gets positive coloration, a red color in the elastic fibers, but even then, after periodic acid oxidation, there is marked variability in the coloration of the various elastic tissues

For example, *ligamentum nuchae*, after the periodic Schiff reagent procedure, gives a weak reaction, by no means intense The arterial elastin gives a positive coloration, but almost invariably around the cortex, around the periphery of the elastic lamellae It is not a uniform coloration and suggests that the medulla is fairly negative and the cortex positive

The connective tissue spreads show a moderately strong coloration with the periodic acid Schiff reagent while skin elastic fibers yield reactions of variable intensity

With "osmication of elastic tissue, the *ligamentum nuchae* gives a fairly strong osmic reaction, the fibers tend to blacken Arterial elastic lamellae give a positive to weak coloration with osmic acid Connective tissue has not been done The human skin, and horse skin, yields a definite blackening of its elastic fibers with osmic acid The same series of relations apply to Sudan black and Sudan IV stained elastic fibers

We have here a series of tinctorial properties of elastic elements some

of which show similarities and some of which show differences

If my point is correct that elastic tissue varies throughout the body, one should be mindful of the particular source of elastic tissue. Having done that, I believe one must also focus upon the age of the elastic tissue in that particular site, because age influences the character of elastic tissue I will develop this point after a few moments

I would like to support this view with chemical data and to introduce these data I would like to shift to discussion of the character of elastin

What do we mean by elastin? What is elastin? I think this is as good a question as, what is elastic tissue? Usually, when we refer to elastic tissue, we actually mean something that is quite refractory to most staining procedures but has an affinity for resorcin-fuchsin, or orcein

Elastin is generally referred to as that which is left over when everything else is hydrolyzed by dilute acid or alkali Elastin is a remarkably insoluble material Most of the methods for procuring elastin involve taking advantage of this profound insolubility; and one of the best methods is the Lowry method, which, as you all know, involves hydrolysis of collagen and muscle, and the various proteins of the tissue, in 0 1 N sodium hydroxide at slightly below boiling temperature (93° to 98° C) for twenty minutes

Following this procedure we find that even at twenty minutes, we do not arrive at a constant dry weight (Figure 16). We have plotted the per cent of the initial dry weight against time in minutes of hydrolysis and find that a constant residual dry weight is not obtained until after thirty minutes of hydrolysis It appears that we may possibly have contaminating materials present in the fraction that Lowry refers to as elastin That this is the case can be determined by two dimensional paper chromatography, taking advantage of the fact that elastin has a peculiar amino acid composition and contains essentially no tryptophane Tryptophane is present, I believe, in a concentration of a few hundredths of one per cent Collagen and many of the other tissue proteins are quite rich in tryptophane, so the presence of tryptophane at any point would indicate contamination of elastin, and indeed at twenty minutes one finds large amounts of tryptophane and many other amino acids not associated with elastin

However, after hydrolysis for thirty or forty minutes, the amino acid composition of the residual material or elastin is quite constant in the human aorta The total amount of elastin in the media of the human aorta hovers between 42 and 43 per cent of the initial dry weight

The significant point is that if we compare the amino acid compositions of samples of elastin from young and old human aortas, they

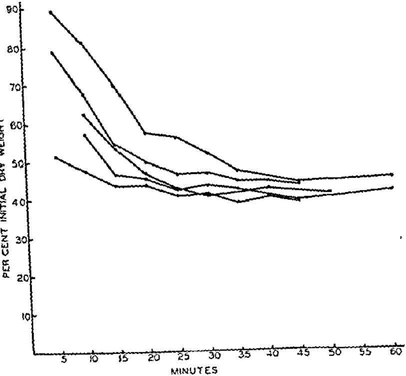


FIGURE 16 Hydrolysis of media of human aorta in 0.1 N NaOH to prepare elastin Note that plateau in hydrolysis is not reached until after 30-35 minutes heating at 98° C

are significantly different Tables II and III amplify the point that I want to make

Here we have analytical data (by bioassay) of the amino acids that make up the bulk of elastin from young and senile aortas Again, this is all human material The glycine, the valine, proline, leucine and isoleucine, are quite constant, but there is a striking change in the aspartic and glutamic acid contents of these two materials Aspartic acid is present in the young in such small amounts, so that it is not even recognizable by chromatography, whereas in the senile, it is present as a very definite and positive spot Other amino acids, it may be noted, also change with age

The essential point is that elastin from an old individual is not the same as elastin from a young one

TABLE II*

Amino Acid Composition of Young and Old Elastin from Human Aortas

Sample	Age	Aspartic Acid	Glutamic Acid	Glycine	Proline	Leucine	Isoleucine	Valine	Amide NH_3	g Ca per 100 g	G N per 100 g† (corrected)
		G N per 100 g N	G N per 100 g N	G N per 100 g N	G N per 100 g N	G N per 100 g N	G N per 100 g N	G N per 100 g N	G N per 100 g N		
Young Aortas	15	0 24	1 32	26 6	11 4	4 71	2 22	13 6	1 43	0 17	15 90
	17	0 32	1 67	25 8	11 1	4 76	2 31	12 3	1 43	0 16	15 75
	19	0 31	1 43	27 0	10 8	4 94	2 27	13 2	1 43	0 18	15 82
	20	0 38	1 63	26 8	11 3	4 84	2 38	12 9	—	0 88	15 35
	Mean	0 31	1 51	26 6	11 2	4 81	2 30	13 2	1.43	0 35	15 71
Old Aortas	55	0 65	2 13	25 6	10 4	4 92	2 46	11 4	1 83	4 58	16 22
	59	1 35	2 70	24 0	9 4	5.01	2.43	11 4	2 30	4 74	15 50
	65	0 68	2 16	24 8	11 3	4 59	2 44	11.9	1 76	6 03	15 19
	65	1 70	3 06	22 6	9 4	4 94	2 60	10 7	2 40	6 61	15 37
	74	1 15	2 33	25 2	10 7	4 73	2 21	12 0	2 22	5 14	15 32
	75	0 90	2 52	23 8	10 2	4 60	2 47	11.4	2 02	8 47	15 75
	Mean	1.07	2.48	24 3	10 2	4 80	2 43	11.5	2 09	5 93	15 56
Pulmonary Arteries	15	0 68	1 84	30 4	11 5	4 68	2 24	12 2	1 51‡	0 13	16 25
	26	0 66	1 99	31 3	10 7	4 96	2 28	13 6	(1 38-	0 17	15 68
	77	0 47	1 40	31 7	9 8	4 53	2 14	12 4	1 76)	0 15	16 27
	92	0 68	1 75	34.5	11 8	4 95	2 47	13 4		0 64	15 78
		0 62	1.75	32 0	11 0	4 78	2 28	12 9	1 51	0 27	16 00

*Reprinted by permission from Lansing, A I, *et al* · *Proc Soc Exper Biol & Med* **76**, *714 (1951)*
†Calculated on a Ca-free basis, assuming all of the Ca to be present as $Ca_3(PO_4)_2$
‡These values were obtained from the analysis of 10 different samples of elastin prepared from pulmonary arteries ranging in age from 31 to 79 years

TABLE III*

Table of Amino Acid Compositions of Various Elastins

Amino Acid	Young 'light' † g N per 100 g N	Old 'heavy' ‡ g N per 100 g N
Aspartic	0.38	1.11
Glutamic	1.83	3.01
Glycine	26.10	21.30
Proline	10.10	9.20
Leucine	4.52	4.76
Isoleucine	2.10	2.33
Valine	13.00	11.50
Alanine	23.18	21.58
Lysine	0.49	1.17
Arginine	1.78	1.35
Histidine	0.15	0.75
Cystine	0.06	0.10
Methionine	0.06	0.35
Phenylalanine	1.73	1.97
Tyrosine	1.45	1.76
Tryptophan	0.06	0.21
Serine	0.29	0.70
Threonine	0.65	1.13
Amide N	2.90	2.86
Total	90.83	90.17

*Reprinted by permission from Lansing, A. I., *et al. Proc Soc Exper Biol & Med* 78, 714 (1951)
†This preparation contained 1.14 per cent calcium and 14.9 per cent nitrogen
‡This preparation contained 6.39 per cent calcium and 12.5 per cent nitrogen

Mirsky Do you hydrolyze the product by some other procedure of your alkaline hydrolysis?

Lansing Yes

Mirsky You do an acid hydrolysis after that?

Lansing: It is about the only way we could get at it

Fremont-Smith: Do you use comparable segments of aorta in the young and old?

Lansing Yes

Fremont-Smith In the old, were you able to use relatively normal looking parts of the aorta, or did you use aortic blocks?

Lansing The first samples we ran by chromatography were without regard to the condition of the intima, so that particularly with paper chromatography one may bring up the matter of arteriosclerotic lesions which do vary with age However, in the later analysis, particularly in

the bioassays, we were careful to select the material to avoid atheromata

Incidentally, the age changes in elastic tissue occur independently of the condition of the intima There is progressive calcification of the elastic tissue with age, whether or not there is atheromatosis

Talbott: Any significant difference between the site at the arch in contrast to the descending aorta?

Lansing: We have not done that (as regards amino acid composition) However, if we can use the appearance of and the degree of calcification of the elastic tissue as a measure of the amino acid pattern the answer would be yes The abdominal aorta shows a more profound degree of age alteration of elastic tissue; there is more fraying and fragmentation of the elastic lamellae and more calcification than in the thoracic aorta

Talbott Go out into any of the peripheral vessels?

Lansing. Yes

Talbott: Is that still different?

Lansing: Yes, indeed

I had not intended to talk about arteriosclerotic lesions but will briefly review some of our observations on elastic tissue changes and their relation to arteriosclerosis.

In a typically youthful aorta, the endothelium is fairly well plastered down on the internal elastica, which is clearly recognizable There are a variable number of elastic lamellae, up to eighty, quite intact, flat plates which stain very densely There are no extensions at all from these elastic lamellae, and an external elastica is not recognizable

With the passage of time, there is progressive breakdown of these plates of elastic tissue, so that one recognizes, let us say, in the thirty-year-old aorta, small tabs of elastic tissue extending from the elastic lamellae in various directions As this age dependent fraying and fragmentation of the elastic lamellae progresses, there is also granulation, so one finds in the aorta of the forty or fifty-year-old individual, granules in broken up portions of elastic tissue. This altered elastic tissue differs from the elastic material in young lamellae, in that it has a profound affinity for calcium

Old elastic tissue in micro-incineration and upon direct chemical analysis, shows massive deposition of calcium This will occur in the abdominal aorta before it occurs in the thoracic aorta The pulmonary artery will rarely show this mineralization, and when it does, it is usually associated with mitral stenosis, or other pulmonary hypertension

The same situation, or a similar one, obtains in the muscular vessels, such as the coronary The young coronary, to describe the morphology briefly, has an endothelium plastered down on the internal elastica.

There is no elastic tissue present in the media of the infantile coronary artery There is also present an external elastica which, in some cases will be multiple That varies quite a bit There is a total lack of elastic elements in this media The inner elastica is intact

In the twenty-year-old individual, and with the passage of time thereafter, there is progressive fragmentation of the elastic tissue in the *elastica interna* Small tags will extend down into the media, and the older the vessel, the greater the accumulation of this type of elastic tissue, which mineralizes, and later aggregates I think the fragmented, calcified elastic tissue may give rise to the calcific bodies that are referred to as Monckeberg's sclerosis in the arteries of the extremities

But the point is that the senile type of elastic tissue, that which is composed of fine fibrils that are frayed and fragmented, that appear granular—that have an affinity for calcium—are different from the elastic fibers that we find in the elastic lamellae of either the muscular or the elastic arteries of youth

This old type of elastic fiber has a more profound affinity for resorcin or orcein It has, of course, large amounts of calcium in it I think I can best summarize the differences between young and old elastic tissue if I go to the next Figure

MacLeod. May I inquire how the other 35 per cent of the amino acids behaved?

Lansing· We can account for 91 per cent of the nitrogen threonine, serine, tryptophane is 0 06 per cent, tyrosine, 0 25 per cent, phenylalanine, 1 7 per cent, methionine, 0 06 per cent, cystine, 0 06 per cent—do you want me to go and give you the whole list?

MacLeod I would like to hear it

Lansing Histidine, 0 15 per cent, arginine, 1 78 per cent, leucine, 0 49 per cent, isoleucine—I think that does it

The amide nitrogen is 2 9 per cent *Hydroxyproline was not included* in the table as published here It runs almost 2 per cent

Ragan How about proline?

Lansing It is a major component It runs 10 per cent It varies between 8 5 to 10 per cent

Mirsky Only two amino acids vary with age?

Lansing· I was 'fudging a little bit on that figure Actually, there are some variations—threonine, for example, goes from 0 65 per cent to 1 1 per cent

Mirsky Is that significant in your acid analysis?

Lansing Yes, that is significant But the interesting aspect of the increase of the aspartic and glutamic acid is that it might account for the affinity of calcium that is recognizable in aging elastic tissue

Angevine What is the relative position of collagen in these arteries?

Lansing: The elastic tissue makes up 43 to 45 per cent of the dry weight of the media of the vessel I say media, because we strip and discard both the intima and adventitia I dare say that the collagen on a rough guess—would be about 15 or 20 per cent of the dry weight That is generous, if we allow for the muscle, which is a major component of the media

Bennett: What is the configuration of the elastic fibrils in these vessels? Are these fibrils cylindrical? Are they round in cross-sectional view, or are they flat plates?

Lansing: The plates or the fibers?

Bennett. The fibers in the muscular vessels that you have talked about

Lansing: You mean within the elastic lamellae, or outside of it?

Bennett Outside of the elastic lamellae.

Lansing. You mean the frayed—

Bennett: The branching

Lansing: They are exceedingly fine—almost entirely of the order of magnitude of one-half micron in diameter. They are cylindrical, but are so small that it is difficult to get an accurate impression of the cross section I will come back to the architecture of the fibers later.

Dempsey They are definitely not plates, though They are not lamellae

Bennett You are coming back to the morphology of the elastic fiber?

Lansing Yes, indeed I was trying to get over some of the generalities that elastic tissue varies throughout the body, that within a given place it varies as a function of age Table IV gives a summary of the variations with age in arterial elastin

This Table outlines some of the properties of young and old human arterial (aorta) elastin In the young the elastic fibers consist of [illegible] water-[illegible] ave [illegible] bers

in the young individual are yellow They are, however, yellow in the old individual, and are also frayed, fragmented, thin strands and granules of elastic staining material; they tend to form clumps

Mirsky: I must interrupt you. By "senile," what age do you refer to, because I am very sensitive on that subject?

Lansing The senile type of elastic fiber can exist in any individual over the age of twenty However, it is present in such an individual, of course, in very small quantities The older the individual, the greater the concentration of this aberrant type of elastic tissue.

Mirsky Since we are all concerned with semantics, I believe it is incorrect to use the term "senile" when referring to this phenomena.

TABLE IV
Resume' of Some Properties of Human Arterial Elastin

	Young	Senile
Physical appearance	Straight, anastomosing threads or ribbons Glistening, refractile, water-clear	Frayed, fragmented, thin strands and granules. Tend to clump, yellow, dull
Tinctorial	1 Not stained by hematoxylin 2 Red color with Congo Red 3 Resorcin fuchsin, orcein, 'Van Giesen' positive	1 Take up hematoxylin 2 Yellow orange with Congo Red 3 Stain more densely with resorcin, etc
Chemical	1 Mineral free 2 Phosphorus-free 3 Amino Acids a) trace of aspartic acid b) 1% glutamic acid	1 Severely mineralized Contains as much as 14% calcium 2 Large amounts of phosphorus 3 Amino Acids a) appreciable amounts of aspartic acid b) 4% glutamic acid
Physical	Dry isolated elastin, has specific gravity less than 1 30	Dry isolated elastin, has specific gravity greater than 1 30

Lansing I am quite willing to delete the word

Mirsky Besides, you must remember my emotional involvement about senility

Lansing. I feel very strongly that senility begins much earlier than is generally accepted

Mirsky I will agree as long as you are willing to say it starts at birth

Lansing. I do not go back that far, I'm sorry I do not want to get into the problem of aging

In regard to the tinctorial characteristics of young and old elastin, the young fibers are not stained by hematoxylin, whereas the old type of elastic fiber does take up hematoxylin or other basic stains in variable amounts This observation may clarify some of the variable statements that one finds in the literature concerning basophilia of elastic fibers

Young elastic fibers stain red with Congo Red, whereas the old type of elastic fiber, the frayed and fragmented type, stains a yellow, or a yellow-orange color

With resorcin-fuchsin or orcein, there is an increased density of staining with advancing age.

The young elastic fiber is mineral free, whereas the old type is severely mineralized and contains as much as 14 per cent calcium on a dry weight basis This is a very marked calcification but does not constitute a concretion, that is, the tissue is still pliable and not readily fractured

The young is phosphorous free, whereas the old contains large amounts of phosphorous, presumably in a proportion that might yield a calcium phosphate crystal of the apatite variety

Gersh: How did you get the 14 per cent figure?

Lansing. By direct analysis and on a dry weight basis

Gersh Did you get any ash in the elastic residue of young vessels?

Lansing None at all. The data indicate that the young fibers are mineral free This is direct chemical analysis.

Hill: You think that calcium is within the fiber, or is it a precipitation or absorption on the outside?

Lansing: I believe it is both. We certainly cannot account for all of the calcium present in old elastic fibers on the basis of the small increase in aspartic and glutamic acids That simply will not do I suspect that what probably happens is a primary linkage of calcium to aspartic and glutamic acids The bound calcium then may combine with a phosphate group which, in turn, combines with a calcium ion, etc , thus setting up a long apatite chain Most of the calcium is an incrustation on the elastic fiber

Gersh. Is it possible that in the preparation some of the material might be washed out from the young elastin, but not in the older specimens?

Lansing: Having checked this in micro-incineration, using a great variety of fixing procedures, including freezing-drying, and having watched the chemical procedures rather carefully, I would say almost categorically that it is absent in the young It just is not there This is not an original observation and is fairly well documented in the literature Normal, young elastic fibers do not contain mineral

Angevine: Do not these differences in tinctorial qualities between the old and young fibers help us to clarify our discusion of this morning relative to the differences in collagen fibrils? We are now talking only of one type of tissue, yet find a great difference in the staining quality, whereas that is exactly what was pointed out in relation to the fine collagen fibrils and the large fibers of different ages

I think that would be supportive evidence, in view of that statement

Hartroft: Could not some of the differences in tinctorial reactions be based on the absence of calcium deposits on the fibers?

Lansing I thought of that as soon as we began to unravel this pat-

tern, and so we studied decalcified sections of aortas and other vessels Curiously enough, the differences in tinctorial reactions persist after decalcification. The increased basophilia is not a function of the presence of calcium.

Lastly, there is a difference in specific gravity, which probably is a function of the calcium When one isolates young elastin and grinds it up to a very fine powder and suspends it in sucrose of specific gravity, 1.30, that elastin will float, whereas similarly prepared elastin from an older individual will settle rather rapidly in sugar of a specific gravity of 1 30.

This may well be a function of the presence of calcium, but at least one can isolate these two types of elastic tissue, and, in isolating the young type of elastic fiber from the old, refine the age differences in amino acid composition

But, again, I would like to comment very briefly upon our definition of elastin I think we will agree that all procedures for preparing elastin assume that elastin is what is left over when everything else is digested This fractionation may be accomplished by using dilute alkali or acid Let us, for example, examine the publications of Gross, from MIT [illegible]
[illegible]
[illegible]
then assumes is elastin You will recall that is the material he studied in the electron microscope before and after trypsin digestion

We have attempted to repeat at least some facets of his work, not so much the electron microscopy, but the preparation of elastin and the action of enzymes on elastin We took elastic tissue from the media of the aorta, and checked the dry weights and composition of the material before and after 1 per cent acetic acid hydrolysis for one hour After that one-hour hydrolysis, we wound up with 93 7 per cent of the starting material In other words, either there is almost 94 per cent elastin in the aorta, or else there is incomplete hydrolysis of the tissue

If one then applies a sodium hydroxide hydrolysis to that same material, the residual dry weight drops to 42 3 per cent This figure is a reproducible value as is also its amino acid composition

What I am driving at is obvious; I do not believe Gross had elastic tissue when he was doing his electron microscopy two years ago

Porter He knows that now, too.

MacLeod. After you extract the aorta, for example, with alkali and heat, does the elastin maintain its tinctorial qualities?

Lansing. Amazingly so The sodium hydroxide extraction in no way interferes with the affinity for the Verhoeff, resorcin-fuchsin or orcein reagents

Figure 17 is a plate from Gross' paper in the *Journal of Gerontology* (1), the original one a year and a half ago, showing the coiling of elastic fibers in the electron microscope. I want to call your attention here to the Gross preparations of elastic tissue that are in the upper figures, to which Gross does not call attention.

Note the suggestion of a twisting of these two major bundles of elastic elements in both of these preparations, just a suggestion of twisting there

The essential point that Gross made was that after exposure of crude preparations of elastin to Armour's trypsin, he was able to characterize in the electron microscope a coiled fibril which was trypsin resistant He postulated that the elastic fiber is composed of a trypsin-digestible matrix, and a trypsin-resistant fibril

This, of course, has been under attack recently, most recently, by Franchi and de Robertis whose illustration I have reproduced in Figure 18 (2) Gross' work, was essentially repeated with and without trypsin digestion, and with sterilized trypsin and unsterilized trypsin Apparently in the unfiltered trypsin, the unsterilized, one gets the fine fibrils that Gross described (See Figure 18).

In sterile trypsin, shown in Figure 19, there are no fibrils, and, lastly, trypsin alone, which is bacterially contaminated, shows the fibrils Franchi and de Robertis concluded that Gross was mistaken in his original observation

The picture, however, may be even more complex than already indicated In Figure 20 can be seen helically-coiled fibrils of the correct dimensions which were made *in vitro* from calcium soaps It is entirely possible that in the bacterially contaminated specimens, calcium soaps existed, both for Franchi and de Robertis, and also for Gross

I noticed a few issues back in the *Journal of Bacteriology*, that after bacteria are exposed to phage, helically-coiled fibrils are recognizable in the electron microscope, and they are, as a matter of fact, almost identical with the helically-coiled fibrils of elastic tissue

Porter I seem to recall that some bacteria have flagellae which coil in this fashion when they are isolated from the cells Possibly, that is what they were looking at

Lansing I bring this up only to indicate that one has to be careful what one calls elastin, and also that the concept of a dual system in the elastic fiber is a bit shaken at the present time It does not seem likely that elastic tissue is digested by trypsin. We are using recrystallized trypsin from Armour and find literally no hydrolysis, no action upon pure elastin Trypsin does not act upon the elastic fiber.

Ragan Is that generally accepted?

Lansing I do not know whether it is accepted, but using every type

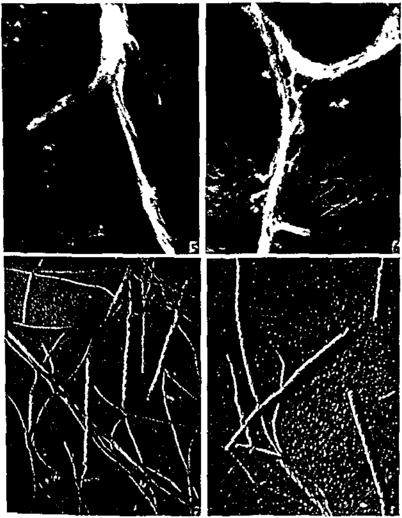


FIGURE 17 Helical coiling in elastic fibers Reprinted by permission from Gross, J *J Gerontol* 5, *343 (1950)*

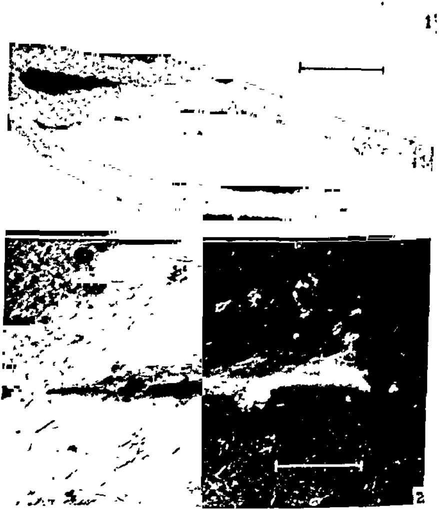


FIGURE 18 Electron micrograph of elastic fibers indicating effects of bacterial contamination Reprinted by permission from Franchi, C M, and De Robertis, E *Proc Soc Exper Biol & Med 76, 515 (1951)*

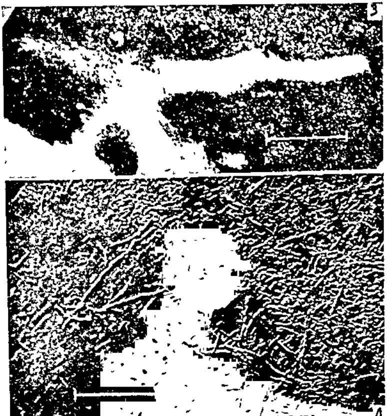


FIGURE 19 Electron micrograph of elastic fibers indicating effects of bacterial contamination Reprinted by permission from Franchi, C M, and De Robertis, E *Proc Soc Exper Biol & Med* 76, 515 (1951)

m
ve

it-
ly used

Mirsky What about pepsin?

Lansing No reaction whatsoever

The difficulty now is to rationalize our observations, which I stated rather categorically, with the generally accepted view that elastic fibers are slowly digested by trypsin I believe it is a function of the purity of the enzyme



FIGURE 20 Electron micrograph of a calcium soap Reprinted by permission from Mottlau, A. Y., *J. Appl Physics* 20, 1055 (1949)

Last year, in the *Biochemical Journal*, Baló and Banga (6), two Hungarians, described an enzyme that they called elastase. It was procured from crude pancreatin by a very simple salting-out procedure Elastase is quite separable from trypsin by salting out, as a matter of fact, in ammonium sulphate

This enzyme has the peculiar property of directly and apparently specifically attacking elastic fibers Baló and Banga report that it is not an hydrolysis, because this enzyme, in causing the dissolution of elastic fibers, did not cause the release of peptides or amino acids. They concluded that elastase converted a fibrous protein into a globular and soluble protein

We, of course, immediately went after this enzyme, prepared it, and found that it did quite what the authors claimed it did Both histologically, and histochemically, and also analytically, we were able to confirm their observations

This enzyme, as I said, is a product of crude pancreatin, which also is very rich in trypsin If one takes commercial trypsin, or the usual reagent grade trypsin that is generally available to most laboratories, one

finds that upon salting-out of that material, one can get a measure of "elastolytic" activity from that trypsin specimen

My guess is that in most previous work with trypsin, the trypsin was contaminated with a measure of elastase This is really a fabulous enzyme It is quite a bit of fun to play with

We have begun to probe the chemistry of the solubilized elastic fibers Upon exposure to the enzyme for thirty minutes at room temperature of arterial elastin, or elastin from many sources, the elastin disappears entirely, and one gets a relatively clear solution that is somewhat opalescent We have examined this clear fraction by electron microscopy with inconclusive results The latter may well be a function of our inexperience with electron microscopy We are not able to recognize definite fibrils

An interesting chemical feature of this "solubilized" material is that as the elastic fibers are digested by elastase, there is the appearance of a creamy fraction which rises in the tubes spontaneously, or which can be accelerated centripetally by centrifugation This material reacts in many ways like a lipid, it takes up Nile blue sulfate and Sudan black, and under the microscope looks like little fat droplets Upon recent chemical analysis—two days ago, as a matter of fact—Dr Robbens was able to recognize sphingo lipids and other lipids in small amounts

This, I think, is particularly interesting, because in the preparation of elastin, the tissue is at least partially defatted The defatting procedure involves refluxing for one hour in acetone followed by refluxing in methyl alcohol, for one hour After this defatting procedure, which totals two hours, the tissue then stands for forty-five minutes in 0 1 normal sodium hydroxide at 98 C, which would certainly give some measure of saponification

After these three procedures, a fatty component of the elastic fiber can still be determined I cannot conceive of this as a contaminant The lipid fraction will probably total somewhat less than 0 1 per cent of the total dry weight of the elastic fiber

The presence of a small amount of fatty material may account for the fact that the elastic fiber has an affinity for osmic acid I do not know It may account for the affinity for Sudan black That is enough supposition But it is rather interesting that it now appears that there is a fatty component to the elastic fiber

Gyorgy Have you tried petrol ethers for extraction?

Lansing We are now using much more rigorous fat extraction to see whether we can remove the fat entirely But more interesting than that, I would like to do elastin preparation without any fat extraction I wonder how much of a naturally occurring lipid is being removed by our procedure, so I would like to go after fresh elastic fibers digested

with this new enzyme It is a crude enzyme, but still used in very small quantities There is no reason to believe that the fat is being added with the enzyme

Hill: Have you tried it on collagen?

Lansing: No We are now beginning to study the reaction of elastase on tissues other than elastic, but *that is in its early stages yet; there is* surprisingly little action—

MacLeod: Is the dissolution, let us say, of the elastic fibril caused by proteolysis?

Lansing. We do not know anything about the enzyme, except that it puts the elastic fiber in solution, and in doing so does not release amino acids or peptides

MacLeod: Or an increase in free amino nitrogen?

Mirsky: In other words, sometimes it is an intact protein

Lansing: Yes Curiously enough, a similar result can be obtained using quarter normal oxalic acid over a period of four to five hours

Gyorgy. With heat, boiling?

Lansing: Yes, with boiling It is a prolonged procedure, but gradually the elastic fibers give way and go into solution

Dempsey The oxalic acid procedure has also a much more damaging effect on other proteins than this enzyme does. We do not like it because it is not good on sections, whereas the elastolytic enzyme will digest away the elastic fibers in tissue sections and leave the other tissues apparently untouched

Mirsky And leave a relatively pure preparation of elastin thereby?

Gyorgy. Any difference between the juvenile and senile?

Lansing We have not yet had time to study that

Gyorgy: Do you use old or young?

Lansing: I have taken sections of elastic fibers from young and old, and there is a suggestion of a difference in rate of dissolution of the elastic fibers I do not have enough data to be certain of this

Maegraith Does the hydrolysis of the elastin give the same results?

Lansing: You mean the quarter normal oxalic acid? We have repeated that only once to make sure that it did work. The procedure as described by Adair (7) in the last issue of *Nature* does work The elastic fibers go into solution, and the resulting solution resembles in a crude way the material that is obtained with elastase.

Holbrook However, you do not know whether it behaves the same, through your various attempts to hydrolyze it or otherwise change it.

Lansing We cannot say However, after the oxalic acid treatment, which is really a weak hydrolysis of the elastin, one can separate an apparently fatty layer from the dissolved elastin by an increase in temperature over 25° C Below 25° C, the material is homogenous, and

as you go above 25°, a creamy fraction separates and rises to the top In a rough way this resembles the fraction that we obtain with the enzyme.

Gersh· Is it viscous?

Lansing Not apparently Adair referred to a material of molecular weight in the neighborhood of 80,000 They describe very little difference between the two fractions that are obtained upon heat separation It is curious that the rate of migration in the electrophoretic field is the same for both fractions—this is something which I do not understand It may be that the electrophoretic rate is the function of a protein, and the lipid is inert But that is rather confusing I do not know what to make of it

Hill: Did you say this acted on the elastin that had the high calcium content, too, the older?

Lansing· The enzyme does, yes The oxalic acid procedure, I do not know

Gyorgy But the enzyme acts on the young one too

Lansing Young and old It draws no distinction, except perhaps in rate There is some suggestion of a difference in rate

Mirsky Does the amino acid composition remain the same, irrespective of whether you use the elastinase or oxalic hydrolysis?

Lansing· We have not checked that

Mirsky You are at least dealing with a purer component

Lansing We will have to purify the enzyme a bit further and characterize the enzyme so that we know what we are putting into the mixture

Mirsky I am worrying about alkaline hydrolysis playing a role somehow in your subsequent hydrolysis of the protein from which you derive your amino acid

Lansing One would think that the 0 1N sodium hydroxide extraction for forty-five minutes at 98 C would markedly damage the elastic fibers, but when dried elastin prepared in this manner is soaked in water for a long time, or in concentrated lactic acid for a short time, there appear under the microscope beautiful elastic fibers that are morphologically identical with what we are accustomed to seeing in fresh tissue sections

Gersh What if they were pulled or stretched?

Lansing They are elastic

Dempsey As they stretch, they become birefringent, just as the native elastic fibers do

Ragan Did Ayer *et al*, (3) isolate their elastin in this manner before it was treated with formic acid? They treated the material with 88 per cent formic acid and showed that in the electron microscope they

could see lengthened and stretched elastic fibers which on neutralization again became amorphous

Dempsey. That was the elastin procedure

Mirsky: Of course, it would be important to determine whether the soluble material is identical with what you have been getting as elastin, in terms of amino acid composition

Lansing: It certainly will be done That really is all I can say about the chemistry of the elastic fiber after exposure to elastase I would like to spend just a few minutes outlining the internal morphology of elastic fibers after exposure to the enzyme

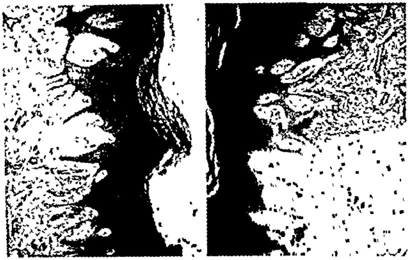
For purposes of orientation I will outline the action of elastase to facilitate interpretation of the illustrations to follow.

As one watches the action of elastase under the microscope (elastic fibers from various sources have been used but for purposes of illustration *ligamentum nuchae* will be described), there is progressive pitting, erosion of the surface of the fiber, which progresses very rapidly This is apparent in five minutes at room temperature. In about ten to fifteen minutes, one sees a dimpling of the entire surface and finally, after twenty minutes, it appears as if the elastic fiber is made up of a pair of coiled fibrils, which still have an affinity for elastic stains

As the enzymatic action progresses, these two coiled fibrils tend to move apart rather jerkily, as if under tension. Finally, one winds up with two separate fibers of reduced dimensions which then proceed again to undergo localized digestion, and each of these again appears to be made up of a pair of helically coiled fibrils. The periodicity of the helix is difficult to photograph, because it involves three dimensions When these finally come apart, there is again an unravelling of a pair of helically-coiled fibrils, this continues until one can no longer see the fibers in the light microscope

The Figures that I will show will not do too good a job of demonstrating the helices They will illustrate longitudinal orientation within elastic fibers, whether they are from the *ligamentum nuchae*, or connective tissue spreads *There will be a suggestion here and there* of the helix Better photomicrographs will be needed to demonstrate the morphology more clearly. In any event, there is longitudinal orientation *within the elastic fibers which appear to be helically-coiled*

Two sections of human skin stained by the Verhoeff procedure are shown in Figure 21, A is a control and B shows elastase digested The control section shows the distribution of the elastic fibers, the coarse fibers are deep in the dermis, the fine ones are just beneath the epidermis After thirty minutes' exposure to elastase, there is almost a complete dissolution of the elastic elements in the dermis. As a matter



A B

FIGURE 21 A Human foot sole incubated in carbonate buffer Verhoeff stain, × 100 B Same as 21A but digested with elastase Note absence of elastic tissue

of fact, one can see the negative images here and there of the elastic fibers

The epidermis is deliberately overstained to preserve any elastic elements that might be present

This, again, shows how little action there is by elastase upon tissue components other than elastic fibers One can recognize nuclei, the epidermal cells here and there can be seen rather clearly

Figure 22A illustrates *ligamentum nuchae* that has been exposed to the enzyme There is a short fragment of the fiber which is fairly intact It takes up the stain very heavily There is a small fragment, about half the diameter of an intact fiber, and in another part of the field we see that the fiber is reduced in diameter It is unravelling at one point into two, and at another into four, with one arm that is out of focus

There is just a suggestion of periodicity that unfortunately is difficult to see Figure 22B shows a few morphologic variations This was a large mass of elastic fibers of *ligamentum nuchae* and here again we have a suggestion of the coiled structure still present in this fiber, which has already divided in two Unravelling at one end of the elastic fiber is apparent

Hartroft At what magnification were these photomicrographs taken?

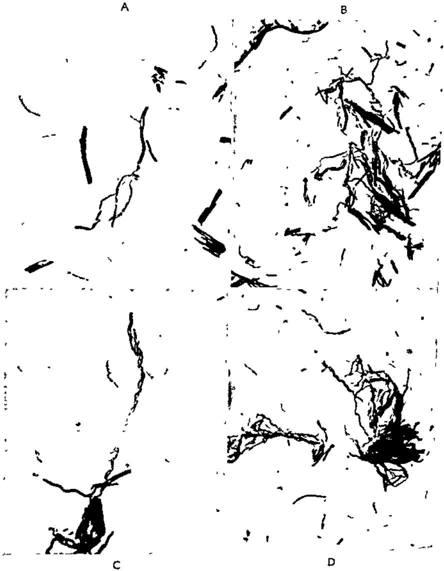


FIGURE 22 Elastic fibers of *ligamentum nuchae* digested by elastase × 280, resorcin-fuchsin stain

Lansing. Two hundred and eighty diameters In Figure 22C this digested fiber in the original photomicrograph looks like a perfect helix. Another in Figure 22D also seems to show a helix There is an obvious longitudinal orientation in almost all of these fibers—another is just beginning to split

Gersh: What are the dimensions of these as compared to those which Gross showed?

Lansing His electron microscopy is at a very different order of magnitude He is working at a few hundredths of a micron, whereas here we are working at 2 to 5 micra

Gersh Could it be that he has the fifth generation of this, or the tenth?

Lansing. It is quite possible That is why I included references to electron microscopy of calcium soaps We know that there is calcium present in many elastic fibers There are also indications that lipids are present Just as a suggestion, it may be that the procedure that he used gave rise to a helically-coiled fibril as a result of the presence of the calcium and the fatty materials It is a possibility It certainly would be a remarkable coincidence to have a helical coiling here in the light microscope and entirely unrelated helices in the electron microscope

Gersh Not if you say they just keep right on

Lansing Yes I think that the Gross story needs further investigation I do not think we have seen the end of it If Gross is wrong, this would be an amazing coincidence

Porter: Except that I think the concept of elastin as being coiled in this fashion is not absolutely new, is it?

Lansing: No

Porter Was it not expected that it would be this way?

Lansing Yes, indeed However, I was surprised to see it I think I first saw it in a dissecting microscope

Gersh. Was that electromicrograph of the soap a direct photo of an impression, or a shadow?

Porter It is an image of shadowed material, but a direct picture

Gersh But it was a shadow I just wondered if the soap would stand up to the beam, or whether it would disappear

Lansing I recall that specimen as being a shadow-cast preparation Figure 22C is an illustration of another digested fiber which, on one end, is showing an unravelling into two, and at the other end two members that are each unravelling in turn In one arm, one gets a suggestion again of the coiling

Figure 22B is a rather good preparation which shows various stages of the digestion There are large fibers splitting into two, and one that is undergoing progressive digestion, again, the coiling is strongly suggested at various points



FIGURE 23 Elastic fibers of loose connective tissue spread digested by elastase Resorcin stain, × 400

In Figure 23A the pattern of splitting and longitudinal orientation is also apparent in unfixed loose connective tissue spreads One can see the longitudinal orientation readily

The fresh specimen of loose connective tissue shows longitudinal orientation of the elastic elements; as does the next specimen which was fixed (Figure 23B) The fiber is unravelled, there is transverse periodicity, and a suggestion of an outer, well-stained periphery. The latter seems to be the case after fixation The general conclusion that *we have been inclined to reach is that the longitudinal orientation is* best demonstrated in fresh material. The enzyme seems to break up the elastic fibers transversely rather than longitudinally after formalin fixation

Also in Figure 23B we have what appears to be a resistant cortex of material in an elastic fiber This is a true resorcin-positive cortex at any depth of focus

Here again in Figure 23D is the transverse periodicity as the fiber divides into two

Figure 23C shows a still more clear-cut demonstration of that periodicity at 400 diameters

Hill Have you done a pH on this?

Lansing: Yes It does not seem to have too much influence on this pattern over a range of pH 6 to pH 9 Do I recall that correctly?

Dempsey I think so Stronger acid does not work so well

Mirsky What is the optimum pH for the elastolytic enzyme?

Lansing The enzyme curiously has an optimum that is 10 2 or 10 4

Dempsey It is a broad maximum

Lansing The plateau ranges from pH 7 to 10, with a secondary peak at 10 4

Figure 24 is included to show that the coiling of elastic fibers occurs *in material that has not been exposed to the enzyme or other chemical* procedures This fiber was found in a section of *ligamentum nuchae* from an old horse Here are a bundle of the large fibers, and between the large fibers is a rather tight helix of two fibers that are interlinked We have since found comparable material in the elastic lamellae of a number of blood vessels Apparently, the coiling of elastic fibers does occur naturally I think that about summarizes what I have

Travell Dr Lansing, have you any observations on differences in tensile strength of the old and young elastic fibers?

Lansing No, I do not We have made no such measurements One gets the impression in looking at them that the tensile strength of the young would be much greater, but that is a morphologist s impression I hope some day soon actually to measure the tensile strength of individual fibers



FIGURE 24 Horse *ligamentum nuchae*, Verhoeff stain, × 1,000.

Travell: How about the distribution of elastic fibers in other ligaments and muscle tendons? Have you made a study of them? We have problems in calcification at special sites in tendons, such as the supraspinatus, and I wonder whether that would bear any relation to this aging process of the elastic fiber and to the accumulation of calcium

Lansing: I think perhaps there might be a connection We have not looked at other ligaments The changes that one finds in dermal elastic tissue are remarkably like those that we have observed in the arterial wall, the so-called elacin of the dermatologists is essentially identical, as near as we can make out, with the senile type of elastin of the arterial wall Both show an affinity for calcium, increased basophilia, and so on down the line

In the literature there have been co...
ment...

... those areas
... is a precedent in the auricles of the heart, which are rich in elastic tissue, and undergo changes and calcification with age

I suspect it is a fairly general phenomenon that elastic fibers when put to stress over long periods of time undergo changes which involve calcification

Travell. Have you any observations as to what accelerates this process?

Lansing: I am a morphologist We have been making, or have just begun to make observations on experimentally treated animals It is much too soon to draw conclusions There is just a trace of a sugges tion—and please take it at no more than that—that thiourac... d young dogs do show these elastic ... s con...

biopsy and ... condition of the coronary artery by assaying the dermal elastic tissue

Hill: I would like to mention provocatively that in hepatic fibrosis, you get a manufacture, as it were, of elastic tissue, and in ... you find a lot of elastic tissue ...

I wonder if ... ones why you s... elastic tissue

Lansing That is rather an unusual event Usually, scarification in volves, of course, the collagenous fiber, and very seldom do we see the elastic element The repair of elastic tissue, on the whole, is rather poor This has been called to my attention before, but I do not know how to interpret it Do you have any ideas Dr Dempsey?

Dempsey No There is another aspect of the elastic fibers in which we are greatly interested That is their fibrilogenesis How are they formed? Where do they come from? We have been searching for a location in which we could experimentally induce the laying down of new elastic tissue, so that we could study the process of formation of it in a favorable place However, except for the obvious, the laying down of elastic fibers in young individuals, we have not been able to think of any good experimental situation in which new elastic fibers would be produced If anyone has any notions on this topic we would be very grateful

Ragan Pulsating heart muscle.

Gersh: Tissue cultures.

Dempsey: Tissue cultures are difficult technically, and there are some limitations to the techniques which have deterred us from trying to set up a tissue culture laboratory.

Ragan: More elastic fibers are laid down when the muscle is pulsating.

Bennett: In a paper given in Washington recently, Dr. Edith Potter of Chicago described certain developmental stages of the lung in the human embryo. I was impressed with her demonstration of elastic fibrils in the bulbous tips of the invaginating septa of future alveolar walls. These structures might serve as source material for newly formed elastic tissue.

Holbrook. I am interested in your solution following the use of elastase. Can you recover elastin from this solution?

Lansing: We do not know. We have not done an amino acid analysis of the solution. The solution, when it dries down, has an affinity for the usual elastic stains, such as resorcin-fuchsin and orcein, the implication being that perhaps a major part, or a significant part, of the elastin remains. That is really all I can say.

Dempsey: We are in definition trouble here, because elastin, by definition is what is left, what remains insoluble. If it is placed in solution it is no longer elastin, by definition, and we do not know what it is.

Porter: Does it reconstitute after acid hydrolysis? Acid hydrolysis is possible of elastin, is it not?

Lansing: You mean the quarter normal oxalic acid I referred to?

Porter: Yes.

Lansing: That is the British work that I cited. I do not know. Adair reports a protein of a molecular size of about 80,000, and that is about all. There was very little said about it in his preliminary report.

Dempsey. The ordinary acid hydrolysis, as it is used here, which destroys elastin and takes it all the way down to amino acids—that is completely destructive, and irreversible.

Porter: The enzyme solution, the elastase solution, does not reconstitute in anything that you have done with it?

Mirsky: Is it a reversible reaction?

Porter: I am only thinking that it is possible to reconstitute collagen fibers from acid solutions.

Lansing: There is a suggestion of that when the solution dries down; there is an appearance of fibers or fibrous-like material which could well be artefact. We have not made a concerted effort yet to characterize the soluble elastin. We do not know much more about it. There is an intense Schiff reaction in that material. When leucofuchsin is

added, the solution becomes bright red, but that again does not tell us too much.

Hill. When this stuff is dried down, you say it takes up the elastin stain, you mean the Verhoeff stain

Lansing: We mean resorcin-fuchsin and orcein

Hill Not the Verhoeff?

Dempsey: I do not believe we did a Verhoeff stain on this dried material It was "solubilized" material that was placed on a microscope slide and watched as it dried down As it dries, it leaves waves of precipitated material behind it This dried residue, if stained with the resorcin-fuchsin stain, or with the orcein stain, stains just like elastin, but we did not try the Verhoeff procedure.

Hill: It is not a chemical reaction, and a lot depends on the differentiation Gaylord and Aschoff (4) did bring this point out of the increase of elastic tissue in hepatic fibrosis

Some people have contested that by saying that there is so much fibrous tissue and round cell infiltration that if you pour the stuff on, you are bound to get a black color, and if they differentiated more—

Lansing One has to be very careful with the Verhoeff procedure to differentiate adequately, or else one will get material other than elastic tissue showing black, which can be interpreted as elastic material The Verhoeff is a good nuclear stain

As a matter of fact, just the other day, in an experiment in our laboratory, we deliberately overoxidized a section of skin by exposing it to periodic acid for a half hour and then applied the elastic stains and found many, many fibers stained which ordinarily do not The immediate assumption would be that we had now uncovered a class of elastic fibers that do not take the usual stain

However, after exposure to elastase, those fibers were untouched, [illegible] hydrolyzed [illegible]

[illegible] (5) By this method, one can take a photograph of tissue unstained and obtain various degrees of density Figure 25 is a photograph of a small artery with endarteritis The vessel is in a section of kidney The tissue is unstained and has been X-rayed, the *elastica interna* is demonstrated very beautifully and shows it to contain something that absorbs or is opaque to X rays

This is presumably not calcification, because there is no evidence of it in routinely stained sections The purpose of our interest in such an instrument is that one can take an X ray of such a tissue and, after removing the lipid, take another photograph and then determine the mass before and after with a densitometer A similar determination

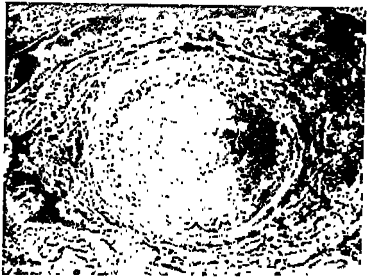


FIGURE 25 Historadiograph of a small artery

could be made in relation to the calcium content.

Holbrook: How old is the artery?

Angeline It was from a young adult

Lansing: My guess is that there probably is quite a bit of calcium in the elastic material that does not show up by ordinary procedures, but with micro-incineration could be brought out.

Angeline: Of course, we know that nerve fibers, which also contain lipid, will show a similar opacity without very much calcium

Lansing: I appreciate your point entirely.

Maegraith: May I ask is there any form of bacterial or other kind of necrosis which will dissolve elastic tissue?

Lansing: There are a number of bacterial preparations that will nonspecifically dissolve elastic fibers I forget the names of the species I played with one last year. In any event, there are a number that have been studied, and they will completely digest elastic fibers, but the enzymes therein have not been characterized

György You related the high calcium content of senile elastin mainly to the higher glutamic acid and aspartic acid Would it not be possible or more probable to relate it to a higher lipid content, which would bring it more in line with arteriosclerosis with this type of calcification?

Lansing I have no objections to the second possibility, except that we have a precedent, an analytical precedent for the first, and none for the second

György. You have for the second too, the fatty acids

Lansing. We have no indication that they change with age in the media of vessels or in the elastic tissue, whereas we do have an indication of the change in the amino acids

György I am asking if you are sure, whether there is a difference or no difference with age, as far as the lipid content is concerned

Lansing: We have no evidence whatsoever for a change with age in the lipid content of elastic fibers There simply are no data there By the way, I have not concluded at all that the dicarboxylic amino acids are the cause of the calcification It is an interesting and suggestive parallel, but it may not be the significant factor in calcification of the elastic fiber

György Lipids and cholesterol fit much more in what we know about senility

Lansing I must take exception there The cholesterol changes with age, or with arteriosclerosis or atheromatosis, are classically confined to the intima, whereas the elastic tissue is equally confined to the media, and unless we can demonstrate a significant change with age in the cholesterol content of the media or its elastic tissue your hypothesis does not hold The limited studies we have done on cholesterol in the media do not indicate significant age change

I think you are transposing what happens in the intima to the media I have no objection to it, but it does not quite add up It is equally possible that the change in the lipid metabolism of the elastic fibers may constitute a source of fat for the intima

György There is no reason to discuss arteriosclerosis here There, of course, the source from the blood circulation has to be considered, as far as arteriosclerosis is concerned

Lansing Which may come up from the *vasa vasorum* and be reflected in the elastic tissue

György I would be interested in the changes of lipid content

Lansing You may be sure that those measurements will be made

Mirsky In view of the fact that with the elastolytic enzyme you can now demonstrate a dissolution of a rather complex structure into a relatively simpler one, is it not possible that your analysis of what was regarded as a purified material may now turn out to be erroneous in that there may be a prosthetic group of which you did not take cognizance?

Lansing Yes It means that much of the chemistry that we have done heretofore has to be repeated with the enzyme

Ragan Would it be fair to say that the difference in elastic tissue throughout the body is also probably a difference in the number of helically-coiled strands that are present, rather than the difference in the actual composition of that tissue?

Lansing Possibly It is a very interesting suggestion

Mirsky. The time factor would be against that. You said the only thing you find different in these senile and presenile elastins is in their reaction to the enzyme That would suggest that perhaps you are dealing with more coiling in the aged elastin than you deal with in the young elastin

Lansing. Well, the old is presumably broken down further, so that one might expect it to go into solution more rapidly, whereas, actually it tends to go into solution more slowly There may be secondary factors operating

Mirsky. You also have calcium there, and you want to know whether it is calcium that is affecting the velocity reaction of the enzyme

Lansing· That was entirely a pilot observation

Campbell. Is there any obvious difference with age in the degree of coiling? Can you see that the old fibers tend to straighten out?

Lansing. They are quite linear when the separation is completed, so I really cannot answer your question

Mirsky· Is there any elastin from various sources in the body which does not react with this enzyme?

Lansing. We have not found it yet, if there is

Dempsey There is one possibility. Now, again, this is a matter of definition As a histologist, the only way I know how to define an elastic fiber is that it is a fiber which stains with resorcin-fuchsin, or orcein, or the classical elastic fiber stains

In the inner ear, there is in the basilar membrane some material which stains with resorcin-fuchsin, and so far we have been unable to digest it with the elastolytic enzyme Whether that is an elastic membrane, or whether we are just in definition trouble here, I cannot interpret

Lansing. Its solubility in dilute acid or alkali is different from usual elastic tissues

Mirsky: If there is anything in the concept of the specificity of enzymes, you would anticipate that by the use of this elastolytic enzyme you could distinguish various types of elastic tissue But since they all except the basilar membrane react with the enzyme, you would have to conclude that they are probably similar

MacLeod No more than you could say that because trypsin digests a variety of proteins therefore they are all the same.

Mirsky Trypsin acts on specific groups

Lansing: They may have certain groups in common Curiously enough, Mr Opdyke, from our laboratory, has demonstrated the digestibility, with elastase, of keratohyalin granules Now we have to determine what relation there is between keratohyaline granules and elastic fibers

Gersh: I have some notes that Banga and his colleagues said at one time that elastic fibers are more firmly bound in the *ligamentum nuchae* than in the aorta There might be different varieties of elastin, in the sense that they may not be necessarily different chemically so much as in the internal forces binding them

Lansing: I believe I made the observation earlier that amino acid analyses indicate that there are differences in the composition of the various elastic tissues

For example, the pulmonary artery has 32 per cent glycine, whereas the aorta has only 26 per cent, and *ligamentum nuchae* varies from that

Hartroft Are these differences also present in young individuals?

Lansing. Yes In *ligamentum nuchae*, there is no change with age in amino acid composition, whereas in the artery, there is, of course a significant change with age The pulmonary artery differs from *ligamentum nuchae*, and the aorta, and shows practically no change with age

Campbell: Is elastin antigenic?

Lansing I do not know I suspect not, it is insoluble

Campbell What about your soluble form after digestion?

Lansing I do not know

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PAIN MECHANISMS IN CONNECTIVE TISSUES*†

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RECENTLY I HAVE been to a number of symposia on pain, in which a large part of the time was consumed in semantics and a discussion of the nature of the pain experience Tonight I would like, at least at the outset, to take for granted that we all know what pain is, and also to take for granted that pain is felt when an adequate stimulus is applied to the appropriate nerve structures and the propagated impulse arrives at the cerebral cortex in the conscious patient That implies a stimulus, an appropriate transmitter, and perception by the subject

We may also assume that pain thresholds exist normally, whether or not they can be measured quantitatively, because with the all-or-none conduction of nerve fibers, if we did not have thresholds, we would be in pain all the time

Another thing that has been shown, and I think we may accept, is that the intensity of pain may vary independent of the pain threshold Hardy, Wolff and Goodell (1) have shown that in the case of injury to the skin, at the site of injury, or area of primary hyperalgesia, there is a lowering of the pain threshold, whereas in the area of secondary hyperalgesia, around the site of injury, there is no lowering of the threshold The same minimal stimulus evokes pain in the secondary hyperalgesic area as in the normal skin Nevertheless, in the area of secondary hyperalgesia, this threshold stimulus produces a more intense pain than it does on the normal skin, and that is in harmony with what we all know from experience, that lightly stroking such an area of secondary hyperalgesia is painful

Although measurements of pain intensity in relation to the pain threshold have not been made for the deeper connective tissue structures as they have for the skin, it seems quite likely that they will exhibit the same kind of behavior

Fremont-Smith: I would like to ask whether the hyperesthetic area around the injured area may not even have an elevated threshold You

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said it did not have a lowered threshold Does it have a normal threshold?

Travell. Approximately the same, I believe

During the past ten or twelve years we have made many observations on pain in human subjects These subjects are of two sorts a) normal people in whom pain was produced experimentally by various procedures, and b) patients who complained of various kinds of pain and who exhibited the enigmatic phenomenon of trigger areas in the myofascial structures.

Some of these patients had rheumatoid arthritis, some osteoarthritis of single joints (the hip or shoulder) and some had joint sprains or muscle injuries, some had cardiac pain, some had neurologic changes attributed to intervertebral disc syndromes, or to metastatic carcinoma, some of them had apparently primary psychogenic pain syndromes, including hysteria But all these people had, as a common denominator, these abnormal foci of pain known as trigger areas

At the present time we know a great deal more about what a trigger area *does* than what it *is*, so I think we will start with our findings and draw inferences later

Talbott: Do you think that the term "trigger area" is really justified as we discuss it so glibly?

Travell: You are involving me in a definition now The term "trigger area" is simply derived from the fact that if you do something at one place, which we call the trigger, then something else happens in another place, which we call the reference zone or target It implies that some relationship exists between two different topographical areas

Why do you object to the term?

Talbott: Does it not also imply—I am thinking purely from the standpoint of some of the things we talk about in physiology—a lowered threshold, or at least hypersusceptibility in a localized area

Travell. Yes, I think these trigger areas are analogous to the sites of injury in the skin, that is, that they represent areas of primary hyperalgesia, and that they do give rise to a barrage of noxious impulses of some sort which affects remote structures But that is not necessarily implied in the term "trigger area

Ragan Does your definition include visceral pain which has a distribution away from the pain stimulus?

Travell You mean, do the viscera contain trigger areas?

Ragan You say you stimulate at one point and have pain at anothe That is your definition of a trigger area Now if you dilate the duo enum and have pain in the lower part of your abdomen is that a trigg area?

Travell. Do you mean, is there a trigger area in the lower part of the abdomen, or one in the duodenum?

Ragan. The pain is referred to the lower part of the abdomen

Travell According to the simple definition I gave you, the source of pain, the duodenum, might be called a trigger area, and the target would be the lower part of the abdomen, the reference zone. The duodenum would serve as a source of pain impulses, but probably these would not result in a sustained pain cycle.

In clinical states, we find that noxious impulses from viscera often lead to the development of trigger areas located within the somatic reference zone of visceral pain These trigger areas then serve as new *sources of noxious impulses, and may take over and carry on the pain* pattern of the visceral lesion (2).

When one distends the duodenum experimentally, the situation would be different from that which exists when there is some pathological or pathophysiological process going on spontaneously in the viscera We should probably restrict the term "trigger area" to apply to abnormal, persistent states of hyperexcitability.

The only way we can study these trigger mechanisms satisfactorily is in the patient who presents this phenomenon In this case, we cannot learn much about the abnormal from the normal

And so most of our observations have to be gleaned, for the present, from the people who develop these states spontaneously So far, we know no way of producing experimentally a complete trigger area and a continuing pain cycle It is a pathological response, or at least it is manifest under pathological conditions, rather than a physiological response which is reproducible

Fremont Smith And is reproducible in the patho-physiological condition

Talbott Is it a general phenomenon?

Travell It is a very general phenomenon in people who have pain *and there are very few people who do not have pain* at some time or other

Of course, nearly all of our observations, in the end, are based on the subjective evaluation of the patient, or of the individual After the satisfying discussions we have just heard on the morphology and the chemistry of connective tissue, with objective findings, our data may seem intangible and hard to get hold of But Dr. Fremont-Smith said this morning that "*science is anything which lends* predictability to data," and these trigger areas and their behavior show a high order of predictability One sees the same things again and again

In the first place, each trigger area gives rise to a predictable pattern

of referred pain, according to its location Figure 26 shows a trigger area in the infraspinatus muscle at the point X (3) It is a well-localized area, and is found only if you look for it If you palpate the general region of the trigger area carefully, you come upon a small tender spot, perhaps as big as your thumbnail, and when you press firmly on it, the spontaneous pain previously described by the patient is reproduced or intensified In this Figure, the place where the patient indicates that pain is felt when pressure is exerted or the trigger area is touched with a needle, is shown by the black shading, cross-hatching and stippling You notice that the trigger area lies entirely outside of the distribution of pain.

The referred pain pattern of the infraspinatus muscle, as shown here, was mapped in a group of about 200 patients, and we have seen it a good many more times since these data were analyzed You can see from the percentages given, that all of these people complained of pain in the front of the shoulder, and about half of them had pain which

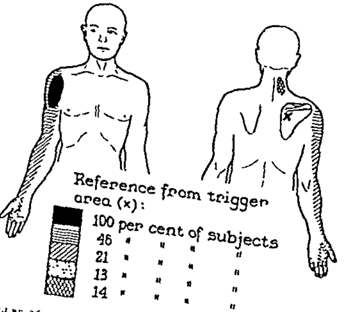


FIGURE 26 Pain reference pattern of trigger area X in infraspinatus muscle (upper portion), as mapped in 193 patients with shoulder pain Reprinted by permission from Travell, J *Mississippi Valley M J* 71, 13 (1949)

extended down over the biceps. Diminishing proportions of the patients complained of pain farther down, in the forearm, back of the hand, or thumb and first two fingers. A small proportion of them also complained of pain high in the back of the neck Again, this distribution of pain was described by the patient when the trigger area was stimulated mechanically Usually the induced pain appeared momentarily and disappeared within a few seconds to a minute afterward

If the patient had pain at the time, constant rest pain, then the existing pain was markedly accentuated, and the increment often did not subside quickly

Fremont-Smith No complaint of pain at point X?

Travell. No complaint of pain at the point X.

In such a patient with shoulder pain and trigger areas in the right infraspinatus, if there is no complaint of pain in the other shoulder, you would probably not find any such areas of deep hyperalgesia in the opposite, the left, infraspinatus muscle

In Figure 26, the black shading shows what we may term the "essential" reference zone of the trigger area at X, since referred pain was felt here by 100 per cent of the subjects, and the further extension of referred pain may be called the "spill-over" (4) The extent of the radiation is apparently related to the sensitivity of the trigger area If the patient has constant rest pain and a great deal of pain, can hardly bear the weight of a sheet on this area and cannot lie on that side, then we observe a wide radiation of pain when the trigger area is stimulated Whereas if he has pain only on motion and not at rest, and we conclude that the threshold is not lowered to the same degree, we see only the pain reference in the essential zone

Fremont-Smith: When you say "lie on that side," you mean on the right side, where the black area is

Travell: No He cannot lie on the right side because the weight of the body presses the trigger area against the right scapula

The trigger mechanism is mechanically stimulated not only by direct pressure, but whenever the muscle is passively stretched The trigger area can be shown to give rise to referred pain also during *active* contraction of the muscle, if the contraction is sufficiently forcible.

Fremont-Smith: That is the muscle in which the trigger area lies

Travell. Yes, the one containing the trigger area. It is easier to set off the referred pain by motion which passively stretches the muscle than by that which involves active contraction

Trigger areas are also intensely stimulated by heat, in the form of hot solutions; diathermy often aggravates the pain They are also very sensitive to prolonged cooling, as by draughts

When I said predictability, I meant that a given region of the myofascial structures gives rise to essentially the same pattern of referred pain in one individual as in another This constancy of pattern implies fixed anatomical pathways which link the trigger area to its reference zone, even though we do not know what the pathways may be

Just as in the case of visceral pain, if you note where the patient has pain and you are familiar with the pattern, you can then use the pattern to find the source of pain

Having just shown that the pattern of referred pain is relatively constant for a given site from person to person, I would like to qualify *this and say that a totally abnormal pattern of referred pain is observed* at times I have seen it only twice in the case of the infraspinatus, out *of many hundred patients with trigger areas in this muscle* One patient who had hysteria, complained of pain deep within the chest, and when a trigger area at the point X in the infraspinatus (*Figure 26*) was infiltrated with procaine, the pain set off by the needle was all referred into the chest A few days later the pain had shifted and the patient complained of pain, not in the chest, but in the shoulder At this time infiltrating the same area in the infraspinatus set off the usual reference to the shoulder and arm In the other patient, the complaint was of pain in the interscapular region, and the first time a trigger area in the infraspinatus muscle was infiltrated, the referred pain matched this complaint The second time it was infiltrated, ten days later, the referred pain was reported to be entirely in the shoulder, the patient had had no spontaneous pain anywhere since the preceding treatment Abnormal reference of pain has also been noted by Jones (5)

If a trigger area is injected with, let us say for the moment, procaine, and the reference of pain is set up by the contact of the needle with this abnormal site, there follows a blocking of this trigger mechanism, which may be permanent, or which may last for longer or for shorter periods of time It often happens that the next time you see the patient the arc of referred pain has been shortened For instance, if you observed a full radiation to the hand from a trigger area in the infraspinatus the first time it was infiltrated, the second time the insertion of the needle might set off pain only in the front of the shoulder, and the third time you saw the patient, the region of the trigger area might not even be tender, and if you inserted a needle into it no referred pain would be set off at all from that site So that we see a change in the state of the trigger area in relation to the clinical relief of pain

If the sensitivity of a trigger area is reduced only to a certain point by procaine infiltration, or perhaps this happens spontaneously without such treatment, the patient may be left with a *clinically latent* trigger mechanism at this site

If you examine patients carefully who have had in the past an acutely painful shoulder with limited motion, even though they have no pain or disability when you examine them, you may still be able to demonstrate the persistence of a latent trigger mechanism You will find one or more tender spots and elicit the predicted pain reference pattern when a needle is inserted into such a spot Needling a normal, nontender muscle does not set off referred pain.

These people are symptom-free for the most part, and yet you find that there is a cropping out in them of the same pain pattern from time to time—after unusual exercise, or minor strain of the muscles in that region, or during extreme fatigue and nervous tension Most people, as they get older—I would not say at what age—exhibit clinically latent trigger mechanisms which can be easily found in relation to stories of old pains and injuries, and these abnormal foci can be shown to lie dormant for years, ready to be activated under conditions of stress.

We have done a few biopsies of trigger areas We did one from the infraspinatus muscle in a man with constant shoulder pain, using only a little procaine in the skin, for local anesthesia When the fascia was exposed, the lightest touch on the fascia, or pinching the fascia, set off the reference of pain to the front of the shoulder and the biceps. The reference was then repeatedly induced with each snip of the scissors, as the piece of muscle was cut out

Gersh What kind of fascia are you talking about?

Travell The surface covering of the muscle. Beyond that, I think we have no evidence as to what particular structure in the muscle is involved or where the nerve endings actually lie Possibly they lie in the blood vessels

Ragan Are there inflammatory cells in this trigger area?

Travell: Not any Trigger areas also appear in joint capsules and ligaments, as well as in muscle.

Figure 27 shows the area of ecchymosis and swelling in an external sprain of the ankle The edematous region in a sprain is not uniformly tender, there is some soreness throughout, but at certain precise spots, exquisite tenderness can be elicited When a needle is put into each one of these spots, a radiation of pain occurs In this patient, as you can see, four trigger areas were found, each with a different spread of pain. From one, (No 4) pain was referred through and through the ankle

I did not mention it when I described the pain pattern of the infraspinatus muscle, but the reference of pain from a trigger area is very often deep into a joint This is not shown in Figure 26, since a black glob on a piece of paper does not give you any sense of depth I want

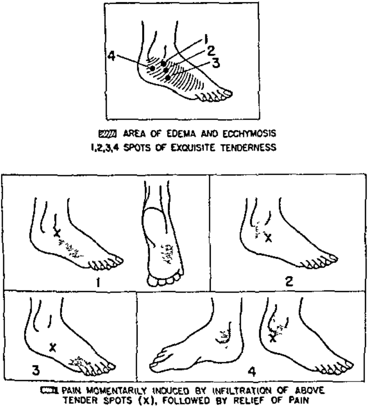


FIGURE 27 Location and pain patterns of trigger areas within zone of trauma in acute ankle sprain, as noted 44 hours after injury in a 43 year old woman

to emphasize that these trigger areas set up referred pain which is apt to center in joints or to give the feeling of being inside the bone

In the case of trigger areas located in fibrous structures about a joint, pain usually is not referred to any great distance from the trigger area in question Trigger areas in the muscles are more likely to refer pain to a considerable distance, though not always, sometimes the reference zone surrounds the trigger area Such differences in pattern have also been reported by Kellgren (6) He injected hypertonic salt solution into various tissues, and found that for ligamentous structures about joints, at least the distal joints of the extremities, the pain reference so induced had a short radiation

Angevine. Have all of these patients been X rayed prior to this time to exclude fractures?

Travell: Yes So far, I have used mainly the "standard" infraspinatus pattern as a basis for discussion This happens to be one of the commonest sites in the body at which trigger areas develop, and it is accessible and convenient to study Now I want to show you some other interesting pain patterns which demonstrate that referred pain from these myofascial trigger areas does not necessarily follow a simple segmental distribution (7)

The concept of the segmental character of referred somatic pain seems not to have been generally questioned, although Sinclair, Weddell and Feindel (8) have pointed out some of the discrepancies Martin (9) states "While referred pain produced by a moderate stimulus is more or less segmental, it may, with a more exciting cause, affect several segments, or on the other hand, only a portion of one segment, or adjacent portions of adjoining segments It is consequently spinal,

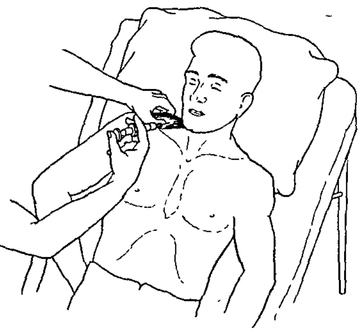


FIGURE 28 Mode of approach for demonstrating and infiltrating trigger areas in the trapezius muscle (anterior suprascapular portion).

but not strictly segmental." This agrees closely with what we have observed

Figure 28 shows the location of a trigger area frequently found in the suprascapular portion of the trapezius muscle With the muscle on the slack, the trigger area can be held between the fingers, and the pain reference set off by squeezing the muscle, or by inserting a needle into the muscle mass at that site This trigger area is most easily approached from the front in the manner shown, with the needle pointing away from the apex of the lung

The pain pattern of this trigger area in the trapezius is pictured in Figure 29, as observed in a large series of patients You can see that pain is referred up into the neck, centering just back of the mastoid process, this is the essential reference zone. When intense, pain extends

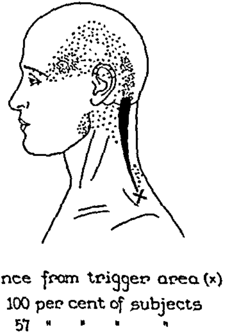


FIGURE 29 Pain reference pattern of trigger area X in trapezius muscle (anterior suprascapular portion), as mapped in 60 patients with neck pain Reprinted by permission from Travell, J *J Am M Women's A* 4, 90 (1949)

to the side of the head, centering in the temple and back of the eyeball This is a reference to the distribution of the fifth cranial nerve, from a muscle innervated by the upper cervical segments Possibly the connection is by way of the *tractus spinalis* of the fifth cranial nerve, which reaches down into the high cervical region.

Kellgren (6) believed that he had mapped the deep pain segments for the trunk and extremities, by injecting hypertonic saline into each interspinous ligament in succession and charting the distribution of referred pain at each level This was done in three subjects, and the pain areas described by these subjects agreed closely. This is a beautiful demonstration of the constancy of referred pain patterns But I have never been convinced that the pattern of each interspinous ligament represents what it is concluded to represent, namely, a deep pain segment, and the recent work of Sinclair, Weddell and Feindel (8) has cast considerable doubt on this

However, let us compare some of the patterns we see clinically with these so-called deep pain segments, which I believe are generally accepted as such Figure 30 pictures the pain pattern of a trigger area,

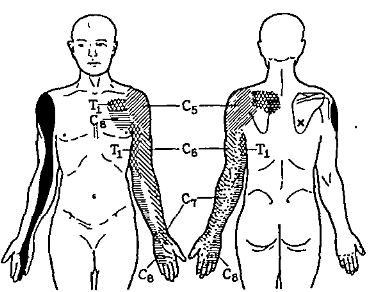


FIGURE 30 Pain reference pattern of trigger area X infraspinatus muscle (lower portion), as mapped in 7 patients The distribution of referred pain is shown in black on the patient's right arm, and is to be contrasted with Kellgren's "segmental pain areas" shown on the patient's left arm

infrequently observed, in the lower portion of the infraspinatus muscle. Incidentally, it calls attention to the fact that different portions of a muscle do not necessarily have identical patterns. As you can see, pain induced from the trigger area radiates to the front of the shoulder, down the ulnar aspect of the arm, and into the fourth finger. If we compare that with Kellgren's charts, shown at the right, you will see that pain cuts through the fifth cervical segment, possibly takes in a portion of the sixth, and then runs down a blank area, skips the seventh except for a small area on the volar surface of the wrist, and reappears in the eighth cervical segment. The pain pattern does not fit the segments.

Figure 31 shows two trigger areas in the *longissimus dorsi* muscle and their respective reference zones. One is in the lower dorsal region, on stimulation it sets off a referred pain in the buttock. A trigger area a bit lower down in the *longissimus* usually refers pain only as far as the sacroiliac joint. You can see that these patterns cut through portions of many segments, without involving any segment completely, the one at the higher level has the longest reference.

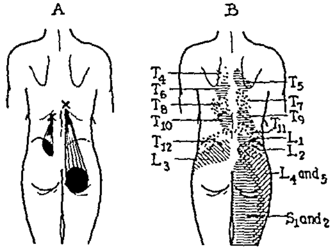


FIGURE 31. (A) Pain reference patterns of two trigger areas X, X in the *longissimus dorsi* muscle at different levels as mapped in 65 patients (left) and 12 patients (right). (B) Kellgren's segmental pain areas.

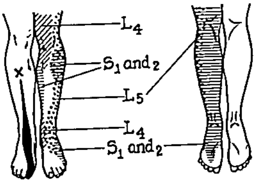


FIGURE 32 Right leg Pain reference pattern of trigger area X in *tibialis anticus* muscle (uppermost portion), as mapped in 18 patients Left leg Kellgren's "segmental pain areas"

Figure 32 shows the pain pattern of an exceedingly common trigger area in the *tibialis anticus* muscle, which sets off referred pain in the ankle, top of the foot, and in the big toe In comparing this pattern with the deep pain segments, we find that the big toe is not included in any segment, and their description is therefore incomplete

Figure 33 shows another type of pattern in which the trigger area and its pattern lie within a single segment. Two neighboring trigger areas in the forearm muscles have very distinctive pain patterns, each one quite different. The one in the long extensor muscle of the middle finger sets off pain centering in the proximal joints of this finger, producing stiffness of these joints The one in the *extensor carpi radialis* muscle gives rise to pain mainly around the external epicondyle of the elbow and in the dorsum of the wrist Both patterns lie within the C_7 deep pain segment

We read a great deal about the lack of precision in the localization of deep pain, but as one studies these patterns, one is impressed with first, the very precise way in which patients can locate their pain, and secondly how well it matches the predicted pattern for the muscles affected.

The patient may not always be able to describe it at first. He can delineate it with great precision by touching it, whereas he may have difficulty in translating it into words. When one sees repeatedly that the patient's delineation of his pain matches the predictable pattern in every detail, one is not so impressed with the term "faulty or poor localization of deep pain"

The pattern, of itself, is faulty, in that it does not tell you where the source of pain is, but the patient can tell you precisely where that pain

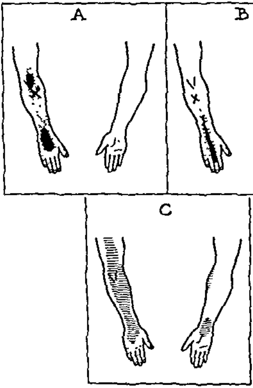


FIGURE 33 Pain reference pattern of trigger area X in (A) extensor *carpi radialis* muscle, as mapped in 15 patients, and in (B) extensor *communis digitorum* muscle (middle finger section), as mapped in 38 patients (C) Kellgren's C_7 segmental pain area

is located if he puts his finger on it This has also been noted by Theobald (10) in an excellent experimental study on referred pain

Figure 34 shows another type of pain pattern which is totally non-segmental This trigger area is located in the suboccipital region and was described in our study on trigger areas in hysteria (11) When stimulated, it gives rise to a prickling sensation with a glove-and stocking distribution which is usually homolateral but may be contralateral, or possibly bilateral

In this subject, the prickling in the palms and in the soles, was repeatedly set off by pressure On infiltration the needle set off a brief discharge of prickling pain in the soles of both feet This was a subject

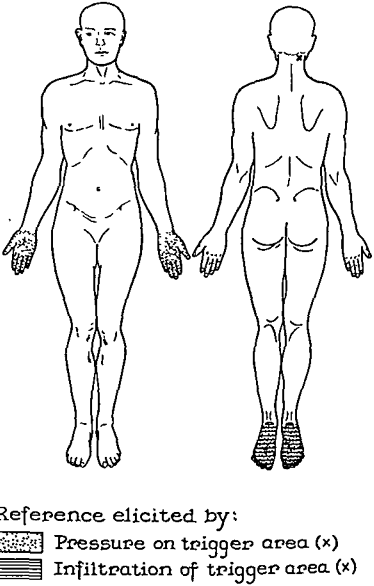


FIGURE 34. Pain reference from a trigger area X in the right sub-occipital musculature as observed in one patient Reprinted by permission from Travell, J, and Bigelow, N H · *Psychosom Med* 9, *361 (1947)*

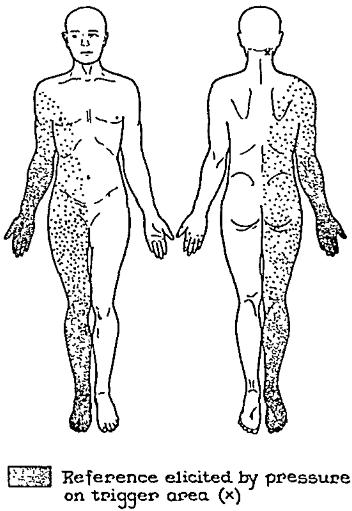


FIGURE 35 Pain reference from a trigger area X in the right sub occipital musculature, as observed in one patient. Reprinted by permission from Travell, J., and Bigelow, N. H. *Psychosom. Med.* 9, 361 (1947)

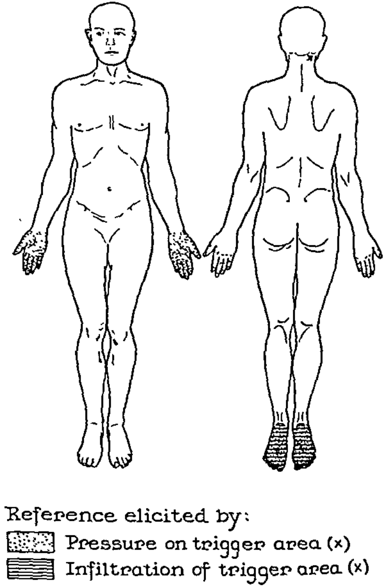


FIGURE 34 Pain reference from a trigger area X in the right suboccipital musculature as observed in one patient Reprinted by permission from Travell, J., and Bigelow, N. H. *Psychosom. Med.* 9, 361 (1947).

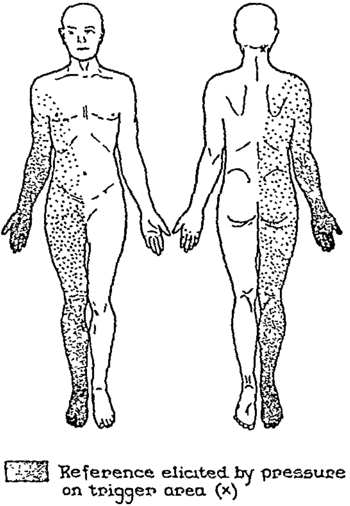


FIGURE 35 Pain reference from a trigger area X in the right sub-occipital musculature, as observed in one patient Reprinted by permission from Travell, J., and Bigelow, N. H. *Psychosom Med* **9**, 361 (1947)

with migraine. Following the block of this trigger area, this pattern could never again be induced, and clinically it disappeared completely, whereas before the injection, it had reappeared off and on spontaneously.

Figure 35 shows another patient with a similar pain reference, elicited from the same trigger area by pressure Here the pattern included most of the lower and upper extremities, and a portion of the trunk on the homolateral side. It was judged to be an hysterical manifestation.

Bennett. Will you have any possible explanation, now or later, for the interruption through a temporary novocaine or procaine block, of a trigger mechanism of this sort which has operated for a long period of time?

Travell: I wanted to talk about that later but we cannot stay away from the question We have not all the answers by any means.

Mirsky: Just to get something clarified, do these patients all come to you or to the clinic with pain in those particular areas?

Travell: That is right

Mirsky Which you then test and find specific trigger areas?

Travell. Which reproduce their spontaneous pain

Mirsky: The point to be clear about is that they do come with spontaneous pain which is then reproduced by the testing

Fremont-Smith: There is another point, and that is that in some of them you find a trigger area which reproduces a pain which they have not complained of, another trigger area

Travell. In checking them over, and in making a map of all the locations where they not only have pain now, but where they have had attacks of pain in the past, you come on these latent trigger areas which match the previous attacks of pain Is that your question?

Fremont-Smith: Yes, and match what other patients have shown

Mirsky. They have always had that pain In other words, you have not induced a new pain area

Travell: Correct

Mirsky. It has always been an area that has been painful at some time in the past of the—

Travell. Practically always Occasionally you have great difficulty in dragging a history of it out of their consciousness They have forgotten it, but if you do not press them on the first occasion, they may tell you on some subsequent visit that they do remember that they used to have a lot of pain there and then recall some special incident years ago But you cannot always get a complete pain history that matches what you find

Bennett: I would like to be sure I understand one point. When patients come to you complaining of spontaneous pain, they complain of their symptom of pain in the area in which you elicit pain from some other trigger area. Is that right?

Travell: Yes.

Bennett: Do they, themselves, come to you complaining of pain which they can elicit from some trigger area?

Travell: No.

Mirsky: They only come from the reference, not the trigger area?

Travell: It is very unusual to have a patient know that he has a trigger area, unless he happens to be a doctor, or someone has already examined him and demonstrated the association.

Talbott: How frequently can you elicit a trigger mechanism in a person who complains of no pain?

Travell: It is almost impossible to find people without some trigger areas and without some pain experience. One of my confreres was of that type, and after years of working with these things, he finally developed an acute pain syndrome from catching his child who fell off the piano. The next day he came in with one angry spot in the lumbar muscles, which had a wide radiation of pain and which kept him from doing everything. He could not lift his medical bag. He could not drive his car. There are a few people who apparently have very few of these pain syndromes and very few trigger areas, but they are hard to find.

Talbott: So your incidence of 100 per cent applies to those persons who have symptoms, probably referable to that area.

Travell: That is right.

Talbott: If you take 100 persons who do not have pain, you might find less than 5 per cent reactors?

Travell: Yes, if you could find 100 people without pain.

Fremont-Smith: When you say 100 per cent there, you mean 100 per cent of people complaining of pain in the reference area — you can induce it by pressing on the appropriate trigger area.

Travell: Yes, with almost 100 per cent certainty. If you possess familiarity with these pain patterns and have the patient sketch exactly where he feels pain, you can usually identify the trigger area from the sketch plus a little information as to what motions cause pain. You can go and put your finger on that one spot, never touch any other one, and set off the pain with what looks to the patient like black magic.

Let us go on to what else these trigger areas do. They set off referred pain when they are stimulated. They also set off deep hyperalgesia, deep tenderness in the reference zone, which appears during the in-

duced pain and which persists longer than the referred pain Cutaneous hyperesthesia sometimes appears too Deep tenderness in the reference zone also accompanies the injection of hypertonic salt solution into normal structures Hypertonic saline is a chemical irritant which reproduces, for the moment, the trigger area; but I have never seen it set up a persistent pain cycle That is something different. The injection of hypertonic salt solution into normal tissues produces a pain pattern, a brief distribution of referred pain, which can be used to check these spontaneously occurring patterns, if you desire

Fremont-Smith: When you inject it into the normal tissue, are you injecting it into what would have been a trigger area, if the patient was having pain, or what would have been a reference zone, if the patient were having pain?

Travell For the moment the hypertonic salt solution acts as a chemical irritant and sets up a barrage of noxious impulses which behaves like the discharge from a trigger area But it fades out quickly.

Fremont-Smith: I still do not understand.

Travell The trigger area is a patho-physiologic process of some kind which persists

Fremont-Smith I do not understand where you are injecting the hypertonic salt

Travell You inject it anywhere you like and the referred pain follows the same pattern as a trigger area at the site of injection would produce

If we look back at Figure 26, on the left we have a normal infraspinatus muscle, not tender The patient has no pain on the left If you inject 0 25 ml of a 6 per cent salt solution at a point in the normal infraspinatus, contralateral to the trigger point X—

Gyorgy. How deep?

Travell. Into the muscle mass—you would set off the same pattern to the front of the shoulder and extending probably down over the biceps—the same pattern as was produced from the trigger area in the right infraspinatus

Fremont-Smith: It is experimentally reproducible with saline.

Travell. Yes, temporarily the pattern of referred pain is reproduced

Bennett: In 100 per cent of persons having no pain?

Fremont-Smith If you were to take people as they came along, such as in this room, how often could you produce referred pain by putting salt into that muscle?

Travell: One hundred per cent There is a difference in the onset time of pain induced from a trigger area and that induced from normal tisues by hypertonic saline

When you insert the needle into the muscle mass and it touches a

of pain it starts with local pain, and then gradually develops, is referred deep into bone and joint and is as disagreeable a sensation almost as can be produced

If you happen to inject hypertonic salt solution into a *trigger area*, or, let us say, 10 per cent Vitamin B_1, or penicillin or some locally irritant material, you have two pains There is first a pain when the needle touches the trigger area If you delay the injection so that you can distinguish these two pains, the injection of the solution will then set up a second intense radiating pain You may occasionally activate a latent trigger mechanism by that process and produce a pain syndrome which lasts for months It is an explanation, I am sure of some of these puzzling postinjection pain phenomena, and it is a good thing to make it a rule never to inject a solution at a site where the needle itself sets off referred pain

I did not mean to talk about that, but it brings out again the impor-

action potentials in the area of pain reference, during stimulation of trigger areas in the infraspinatus, and also during injection of hypertonic saline into the normal infraspinatus to set off the same pattern During the induced pain, resting action potentials appeared in the reference zone, in the deltoid and the biceps, while at the same time they were absent in adjacent muscles, in the triceps Similar electromyographic activity in the reference zone has been observed by Kellgren (13) and by Simons and Wolff (14), using hypertonic saline to induce pain

Vasomotor and other autonomic effects appear during the pain reference which are limited to the reference zone For example, occasionally, in dealing with trigger areas of marked hypersensitivity, pressure will set off a wave of gooseflesh due to pilomotor stimulation, or you may see gross sweating, beads of sweat that break out only in the reference zone The skin temperature may be continuously lowered by one or two degrees in reference zones if the patient has continuous pain, as compared with the contralateral area, and transient cooling of the skin in the reference zone may accompany pain induced from a trigger area

Fremont-Smith: That could be sweating, could it not?

Travell: It could be, yes. On the other hand, the blocking of a trigger area may be followed by gross vasodilatation in the reference zone when one is dealing with high intensity discharges from a trigger area (11).

For example, Figure 36 shows the spread of pain set off in a patient who had a very hypersensitive trigger area in the trapezius muscle, and severe headache. On infiltration of this trigger area with procaine, there was an intense discharge of pain to the neck, the occiput, the temple and the ear on the same side, and almost immediately afterward, an intense prickling pain the opposite ear. Such a crossed reference of pain from an exceedingly hypersensitive trigger area is not uncommon. The pathways for the mirror-image reference of pain have been studied by Ray and Wolff (15).

The most interesting thing about this patient was that almost immediately after the injection, she complained that the right ear, on the opposite side, was burning. The entire external ear was lobster red and hot to the touch. The vasodilatation in that ear persisted for four hours. This is a rather extreme effect, in that any visible vasodilatation usually is much more transient, a matter of a minute or so, and is not observed in connection with trigger zones of low intensity.

Dr. Nolton H. Bigelow and I (12) attempted to study vasomotor

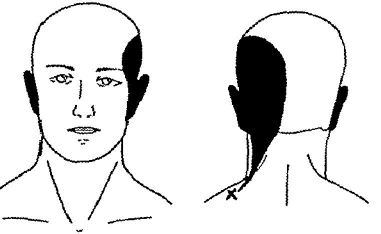


FIGURE 36. Pain reference (black) from trigger area X in trapezius muscle as observed in one patient. Reprinted by permission from Travell, J., and Bigelow, N. H. *Psychosom. Med.* 9, 358 (1947).

changes in the reference zone by placing a tambour on the temporal artery and stimulating trigger areas which set off referred pain to the temple and forehead A typical experiment is illustrated in Figure 37. We used subjects who had headache from time to time, and we studied them in the stage when the trigger mechanism was of latent clinical activity That is, they had no spontaneous headache at the time of the experiment, but pressure on the trigger area, usually in the trapezius muscle, as shown in Figure 28, induced pain with the characteristic pattern that you should recognize now (Figures 29, 37). Changes in arterial pulsation were registered by a beam of light reflected from a tiny mirror on the tambour

To understand these results, I should tell you that sustained pressure on a trigger area first sets off the referred pain, but then if the pressure is continued longer, it will often block the trigger mechanism This effect may depend on the production of ischemia in nerve fibers, which conduction block We do not know what the mechanism is

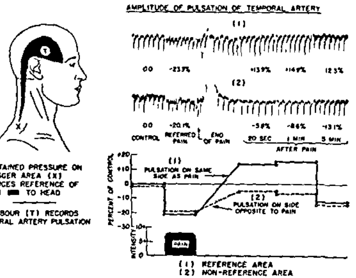


FIGURE 37 Typical experiment showing vasomotor changes in the reference zone in relation to stimulation and partial blocking of a latent trigger area by pressure Actual records of pulsation for homo lateral (1) and contralateral (2) temporal arteries are reproduced above, and a graph of the percentage changes in terms of their control amplitude is shown below

In these experiments, which were never published in detail, pressure was sustained until the referred pain disappeared. At that instant, the subject signalled with his hand and the pressure was released. Coincident with the end of pain, the recording from the reference zone showed an increase in the amplitude of pulsation which amounted sometimes to 15 or 20 per cent of the control amplitude. Remember that these were latent, rather insensitive trigger mechanisms. When the experiment was repeated and pulsation was recorded from the contralateral temporal artery, it can be seen that no increase in amplitude occurred.

Figure 38 shows, in the upper left quadrant, a composite curve obtained by averaging the results of seven such experiments with recording from the reference zone. In every instance, with the subsidence of pain, this transient increase in pulsation appeared in the reference zone, whereas in five experiments none occurred in the contralateral side, a nonreference zone (Figure 38, lower left quadrant).

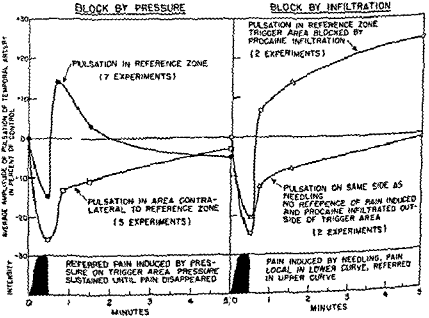


FIGURE 38. Effect of blocking a latent trigger area on arterial pulsation measured within the reference zone and in the contralateral control region. Note that disappearance of referred pain (black) is followed by vasodilatation limited to the reference zone.

In two experiments, when a needle was inserted into the trigger area, as shown by the immediate reference of pain, and procaine was infiltrated, a more sustained increase in the amplitude of pulsation in the reference zone was obtained, extending over at least five minutes. The results are shown in the upper right quadrant of Figure 38.

In the lower right quadrant of the chart, a control is shown. The amplitude of pulsation of the *homo*lateral temporal artery was measured in the same individual prior to the above procaine infiltration. When the needle was inserted into the trapezius muscle, it did not hit the trigger area, as shown by the fact that no reference of pain was induced, and the procaine was infiltrated outside of the trigger area. This procedure caused no vasodilatation in what would have been the reference zone, if the trigger area had been hit.

The older theories of the mechanism of referred pain are based on a direct spread of excitation in the neuraxis, but Roberts (16) has recently formulated the concept that ischemia of nerves in the reference zone of visceral pain may result from reflex constriction of the *vasa nervorum* and that this may explain some of the phenomena of referred pain. His diagram (Figure 39) is very interesting to us as it superimposes a vascular component on the neural mechanisms of referred pain. In this diagram he indicates that noxious impulses from a myocardial infarct, traveling over the visceral afferents to the lateral horn of the cord and to the reference zone via the sympathetics, may constrict the *vasa nervorum* that supply the nerve fibers in this zone. This would result in a diminished blood supply to the nerves, and some degree of ischemia. Bronk (17), Kugelberg (18) and others have shown that certain conditions of ischemia may cause spontaneous firing of nerve fibers which in turn, could give rise to motor effects in the reference zone, as well as sensory patterns, bizarre patterns of pain reference.

This does not rule out the old theories of the mechanism of referred pain—misinterpretation by the cortex, development of a central excitatory state, and so on, but it adds another possibility as to the origin of pain impulses derived from a reference area. It is a very interesting concept which our work would tend to show might apply.

This concept is of further interest to us since there is some evidence (11, 19) that the viscera, including specific areas of the brain and spinal cord, may behave like reference zones for myofascial trigger areas and may be subject to their vasomotor influence. For example, the glove-and-stocking reference pattern that I showed you (Figures 34 and 35) could be produced by a specific vasoconstriction of the posterior spinal arteries and ischemia of the dorsal columns of the spinal

SCHEME SHOWING CIRCUIT FOR REFERRED CARDIAC PAIN ON BASIS OF SPASTIC VASO-NEUROPATHY

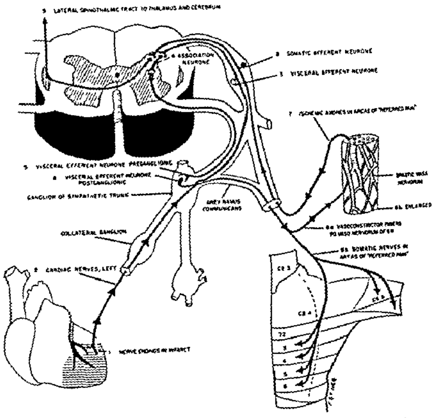


FIGURE 39 Robert's schematic diagram of pathway of noxious impulses from the heart to the *vasa nervorum* within the reference zone of visceral pain Reprinted by permission from Roberts, J T.: *Am Heart J* 35, 369 (1948)

cord, the sensory tracts of Goll and Burdach, which these arteries supply (11)

I should say something about other effects which accompany high intensity discharges from trigger areas, other changes which Campbell and Parsons (20) have termed "the concomitants" of referred pain By way of illustration I will describe to you the "sternomastoid syndrome"

As in the case of the trapezius muscle, the sternomastoid muscle can be put on the slack, lifted off the underlying structures of the neck and held between the fingers so that it can be readily compressed or infil-

trated (Figure 40). In a series of patients, the pain pattern of the sternomastoid muscle was mapped, the composite pattern from all its regions (Figure 41) You can see that the pattern has certain accents of more intense pain—a spot of pain on the forehead at about the hairline, pain in the ear, in the posterior mastoid process, pain in the throat and chin with a wide distribution through the face Trigger areas in this muscle are probably responsible for quite a number of the so-called "atypical" facial neuralgias

When these trigger areas are of high intensity, they produce certain concomitant effects, often without much or any pain The patient veers to one side. If the trigger mechanism is in the right sternomastoid muscle, he staggers to the right If it is in the left, he veers to the left The patient is surprised to find that he cannot gauge distances and bumps into things He starts to go through a doorway and unexpectedly walks into one side of it He also complains of vague visual disturbances, a "mist before the eyes" or some blurring of vision

If this trigger mechanism becomes more sensitive, the patient develops spinning sensations, vertigo, intermittent double vision and even

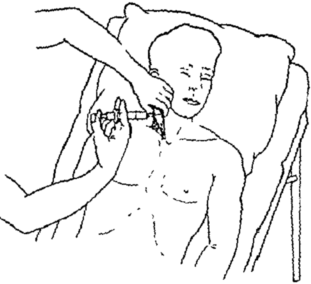


FIGURE 40 Mode of approach for demonstrating and infiltrating trigger areas in the sternomastoid muscle

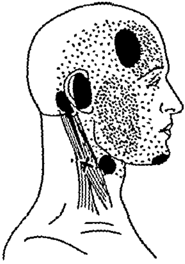


FIGURE 41 Composite pain reference pattern of trigger areas X in both sections of the sternomastoid muscle, as mapped in 28 patients Black shows predominating areas of pain Reprinted by permission from Travell, J, and Bigelow, N H *Psychosom Med* 9, 360 (1947)

nystagmus And I have seen the nystagmus and the entire syndrome, which would ordinarily be regarded as a cerebellar, or possibly a vestibular disturbance, abolished immediately by infiltration of the trigger areas in the sternomastoid muscle This may be permanent or the syndrome may recur, depending on what the etiology is, what is the source of impulses activating this abnormal trigger mechanism.

A number of patients with a sternomastoid syndrome developed chest pain, mainly substernal, resembling the pain of coronary insufficiency The substernal chest pain in these patients was terminated by blocking trigger areas on the face of the sternum, it apparently originated in the rudimentary sternalis muscle (Figure 42) The composite pain pattern of this muscle is shown in Figure 43 Embryologically, the sternalis is thought to have an origin, at least in part, from the sternomastoid muscle One wonders whether the reverse may not be true, that in cardiac pain, in which trigger areas usually develop in the sternalis muscle, within the somatic reference zone of cardiac pain (21, 22), whether the pain referred from the heart to the throat, jaw and face, may not be mediated through this connection.

I don't want to talk about therapy, the therapeutic application of procaine infiltration (23), except as it helps us understand the pain mechanisms that we have under consideration.

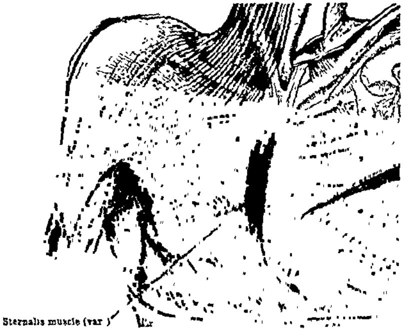


FIGURE 42 Sternalis muscle Toldt, C *An Atlas of Human Anatomy, 2nd ed, Part III* New York, The Macmillan Co, 1928 (p 282)

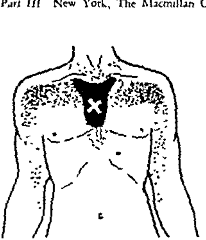


FIGURE 43 Pain reference pattern of trigger area X in the sternalis muscle, as mapped in 10 patients with substernal pain

The dramatic clinical result consists of two parts: a) subjective relief of pain, and b) objective improvement in the range of motion

Figure 44 shows these typical effects following local block of trigger areas in the masseter muscle in a patient with acute trismus. This was a medical student, and we were making some measurements of the normal maximum aperture of the mouth, so we had a previous record He went home for his Christmas vacation and had a wisdom tooth extracted on the first day of his vacation That night he developed intense unilateral facial neuralgia, and was unable to open his mouth; the aperture between the incisor teeth was only about 1.5 cm. The restriction persisted until he was seen a week later, at which time the trigger areas in the masseter muscle on the painful side were infiltrated There was immediately a small improvement in motion, and practically total relief of pain At intervals of several days, the infiltration of trigger areas was repeated Each time there was an immediate increase in the aperture The aperture changed very little between treatments.

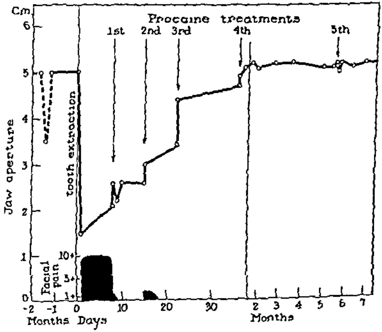


FIGURE 44 Response of pain (black) and limited motion (——) to procaine infiltration of trigger areas, in a case of acute trismus following tooth extraction.

That is the customary response to the blocking of myofascial trigger areas: immediate relief of referred pain, and a steplike release of the restricted motion. This is a typical response

An interesting observation came out of this This patient could not tell how wide open his mouth was unless he put his fingers in to measure it. He did not feel the restriction. At the maximum stretch it always felt wide open to him The proprioceptive impulses that gave him that information apparently derived from the antagonists of the masseter muscle, and the resistance to stretch would inform him that his mouth was wide open when it might be open only 2 cm

Angetine. How wide do you suppose it would have opened in thirty days without procaine?

Travell: A good many of such cases gradually become symptom free without treatment The most striking thing about the case I described is the *immediate* relief of pain

I have, on the other hand, seen a number of patients who had had an acute trismus following tooth extraction, and years later, maybe three years or thirty years later, they still showed a restricted opening of the mouth to 3 or 4 cm But such people do not know that it is restricted unless there is some disability that directs their attention to the limitation of motion such as trying to eat a three decker sandwich and finding they cannot bite it

Bennett. What degree of improvement do you get in these patients with long-term involvement following injection?

Travell. I would say that it is not entirely predictable I have seen limited motion of the neck or the shoulder present for ten or fifteen years, completely restored to normal with one treatment However, the chances are that it will take a good deal of treatment, that there are many trigger areas, in many muscles in the region Other shortened parallel lines will be uncovered as you release the one that is the tightest

Bennett Do you get atrophy of a muscle that is not used because of spasticity?

Travell You do not get any atrophy at all, and that is a very interesting point These muscles that have been relatively inactive for periods of years show no atrophy on physical examination, and on blocking the trigger mechanism, they will start at once to function perfectly

The lack of atrophy is surprising since the muscle that contains a trigger area will not take a load well, it exhibits a pseudo-weakness It is partly shortened all the time Perhaps it needs a longer initial length for a strong contraction Anyway, the weakness is often mistaken for atrophy

A trigger area in the *vastus medialis,* which is the main muscular support of the knee, will often produce a situation in which the knee buckles without pain, when the person steps up a step or places an unexpected load on this muscle The contraction of the muscle is in some way *inhibited in advance of pain.*

I want to tell you now about the blocking of trigger areas by physiologic saline, without procaine, which brings us back to Dr. Bennett's *question as to how a temporary local anesthesia can permanently interrupt* a trigger mechanism

Figure 45 shows an acute knee sprain in a boy who fell down a flight of subway stairs Thirty hours after the injury, the range of motion was very limited The knee could not be straightened. The boy could not walk up stairs The knee was swollen and any motion was painful. X rays were negative. Two tender spots over the inner aspect of the knee joint were infiltrated with a little physiologic saline solution, and immediately afterward the range of motion had increased markedly, as shown by the broken-line circumference between the arrows It was not 100 per cent, in that normally knee flexion should bring the heel down directly against the buttock The boy was kept on limited activity

When he was seen two days later, the range of motion, as shown by the stippled area, (Figure 38), was a little better than it had been di-

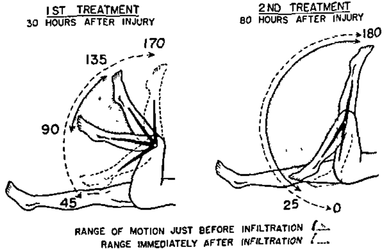


FIGURE 45 Effect of infiltrating trigger areas with physiologic saline on limited motion in acute knee sprain, in a 14 year-old boy

rectly after the first treatment Immediately after the injection of an additional trigger area, the range of motion was perfect

In this case, for experimental reasons, physiologic saline was used instead of procaine, and an excellent therapeutic result was obtained Trigger areas may be blocked not only by physiologic saline, but even by dry needling, if the trigger area is thoroughly peppered with the needle (24)

I think that in clinical experience, the order of efficiency would be procaine first, saline next, and needling last Theobald (10) reached a similar conclusion on the basis of observations on referred pain induced from the uterus by direct electrical stimulation He found that at a mild intensity of stimulation, needling the reference zone in the lower part of the abdomen would suffice to abolish the referred pain When the intensity of the current was increased somewhat, needling would not abolish the pain, but physiologic saline would If still higher intensities were used, relief of pain was obtained only with procaine

Mirsky: The injection being at the reference point?

Travell. In the reference zone of uterine pain

Mirsky: When you talk about treatment here, is it always in the reference zone or in the trigger zone?

Travell: In the trigger zone The confusing thing is that within the somatic reference zone of visceral pain, trigger areas may become secondarily established When this is the case it may be possible to abolish cardiac pain, for instance, by infiltrating the trigger areas located on the face of the sternum or in the precordium, within the reference zone of cardiac pain

Mirsky As if they were secondary trigger areas

Travell Yes Whenever there is a barrage of noxious impulses for a long period of time—for instance, from trigger areas in the infraspinatus—secondary trigger zones are apt to appear in the reference zone—in this case in the anterior deltoid and forearm supinator muscles, and these may have to be handled separately

Fremont-Smith In that connection, is it not your practice in treatment to deal first with the secondary foci, and then come only last to the—

Travell You will usually uncover the trigger areas in the reverse order of their development The more recent secondary foci will, at the time you see the patient, often be the major source of pain, so you pick them off and work backward The more recent trigger areas seem to be easier to find

If you could identify the initial one first, perhaps that would be the best way to get at it

We have another technique for blocking these trigger areas, in addition to the local infiltration procedure.

Mirsky Y[illegible]

then you tol[illegible]

I believe we would all interpret you to mean that you actually blocked the passage of impulses along the nerve.

Travell That would be a justifiable assumption.

Mirsky. I know of no physiologic mechanism whereby the blocking of impulses along a nerve can result from the injection of saline

Travell That would apply to needling also

Mirsky. So although there may not be a perception of pain, that is, the reference zone may no longer respond, is it proper to talk about the end result as if you were talking about a process? When you speak of "blocking," you refer to the process whereby impulses are inhibited

Travell That has bothered me too. The fact is that if you thoroughly needle a trigger area, of deep hyperalgesia, you not only abolish the pain and hyperalgesia in the reference zone, but you also abolish the local sensitivity at the site of the trigger area, usually within a matter of half a minute, or a minute, just as in the case of procaine solution

Wier Can you block the trigger mechanism with external pressure?

Travell If you sustain the pressure long enough and hard enough, for a matter of about two or three minutes, — provided always the patient will tolerate it, — you can often block the trigger area by pressure alone.

Mirsky May I get something clear? In every instance where you are going to stop a response to a trigger area, you get intense pain during the process that is going to result in the cessation of pain, since it is a trigger area

Travell That is correct, and we may be dealing with a postimulatory depression phase

Mirsky In other words, the patient does get intense pain.

Travell. Yes, a very intense momentary pain

There is another procedure in addition to local infiltration that we have been interested in, and that is ethyl chloride spray. When applied to the skin over trigger areas, this is often effective in blocking them, without itself producing pain (3, 4, 19).

Ethyl chloride spray probably falls into the category of counterirritants The interesting thing is that in using this spray, we apply it very lightly, and in a rhythmic manner which produces relatively little cooling

The use of this spray for the relief of painful motion is not new (25, 26) It has been in vogue for nearly twenty years in various parts

of the world, but it has usually been applied up to the point of frosting the skin, with the idea that profound local anesthesia is necessary But actually, no such effect, that is freezing, need be produced any more than local anesthesia in the ordinary sense is essential to the action of procaine in blocking trigger areas by direct infiltration

For lack of a better term, I will continue to say "blocking"

W'ter Can you do that with water?

counterirritants occurs both with the application and the withdrawal of the stimulus. Intermittent cold has been found by Gammon and Starr (27), in experiments on counterirritants and deep and superficial pain, to be more effective than continuous cold We found that the intermittent application of a cold seltzer-water spray may relieve deep ischemic pain (28), but the effect is not as precise, in that I think there is a damping of the rate of change of the temperature of the skin

Another factor in the effect of ethyl chloride spray may be adaptation

interruption of the flow of a certain kind of impulses from the skin So we have two features in the mechanism of action of ethyl chloride spray, in the manner that we have applied it One is intermittent cold and the other is intermittent touch

An analysis of the results obtained by ethyl chloride spray in forty cases of so-called "stiff neck" is shown in Figure 46 The pain and stiffness had been present for from one to three days prior to treatment We called this 100 per cent pain and stiffness, whatever it was at the time treatment was instituted The spray was applied over the trigger areas in the neck muscles, the major one usually being in the trapezius A large proportion of these patients (curve 1) showed complete and immediate relief of pain, and restoration of motion to normal *during* the spraying A certain proportion of them (curve 2) showed marked, immediate improvement but an hour or two later there was some recurrence of the stiffness and pain, but not so bad as it was in the beginning, and in a few hours the neck motion was perfectly normal

These two groups represent about two-thirds of the cases Then there was anoth

motion improve

of motion was

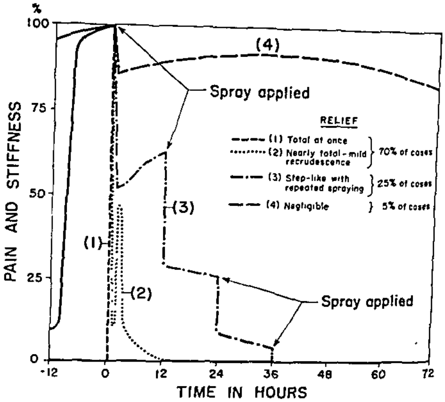


FIGURE 46 Types of clinical responses to ethyl chloride spray in 40 cases of acute "stiff neck" Pain and limited motion are taken as 100 per cent at time of first application

during two or three days, a step-like release was obtained very similar to what we saw with the use of procaine infiltration (Figure 44)

There were a few patients (curve 4) in whom the spray was apparently ineffective Such cases can often be effectively managed by direct infiltration of the trigger areas

We tried to obtain an objective index, some painful lesion that we could see, to measure effects of the spray, and so we decided to study its effect on burns We (30) produced about one hundred thermal burns in human subjects The burns were made in pairs, at comparable sites on opposite extremities, one extremity being treated with ethyl chloride spray in the manner that I have described, and the other serving as control We used the Hardy-Wolff-Goodell pain-threshold apparatus to produce these burns, and we found that pain, as you would expect—

Fremont-Smith: How large was the area burned?

Travell: Each burn was about half a centimeter in diameter, slightly smaller than the aperture through which the skin was exposed to the heat (light) source

Pain and secondary hyperalgesia were relieved to a very marked degree by spraying with ethyl chloride These results, like those obtained in acute joint sprains, indicate that pain in traumatized tissue is not proportional to and is not dependent on the extent of the tissue damage, because pain can be abolished at once, while the tissue damage, which we can see, persists

The size and intensity of the burns was also reduced by ethyl chloride spray The spray was applied about one minute after burning and twenty minutes later there was a marked diference in the zone of erythema around the site of injury Several hours later the burns were obvious on the control arm, but were scarcely visible on the sprayed arm

Mirsky The spray was on the site of injury?

Travell The spray covered the whole volar aspect of the forearm, and covered a line of burns, usually three, which were made in rapid succession.

When the spray was applied as long as ten minutes after burning the skin the size of most of the burns was materially reduced

I have not really answered your question, Dr Bennett, about the mechanism of action of procaine infiltration in blocking trigger areas, but at least I have told you what we observe and what the results are

Bennett Do you know the answer?

Travell No Possibly the interruption of the pain cycle depends on a temporary state of poststimulatory depression following strong stimulation of the trigger area by the needle or of skin receptors by the spray Or perhaps the afferent discharges disrupt an abnormal electrical rhythm, a hypersynchronous beating of locked neurons that maintains the pain cycle, as suggested by Gerard (31) to explain the phenomena of causalgia

At any rate, central effects, at some level not necessarily spinal—it may even be cortical—must play a very important role in it I do not see what explanation there can be for the lasting effects of ethyl chloride spray on deep pain other than central effects

Fremont-Smith Does not the word 'blocking' or "interrupting" suggest that the pain is a reverberatory process in which the trigger zone, the reference area, and perhaps in connection with viscera, deeper areas, are involved in a self-perpetuating circuit in which the pain is maintained, and that what happens here, by the therapeutic application, by

a mechanism not understood—this circuit is broken, and the pain stops

It would seem that, in connection with the burn, this would be fairly clear That is, a burn is not just one pain; it continues and there must be something that keeps it going; then, a sudden spray with ethyl chloride and it stops which suggests that some kind of circuit has been interrupted

Travell· That is the inference one arrives at Leriche (32) advanced that concept when he gave the impetus to the clinical use of local procaine infiltration for relief of pain, and Livingston (33) crystallized it

Fremont-Smith· This is *not my idea*

Mirsky· I could not accept that to mean a block.

Fremont-Smith. It is not block in the sense—

Mirsky· It is an interruption of the patient's response, the patient's response being that of pain. Until we have methodology which can establish without question what we are talking about, I believe it wisest to be precise about our language and what we are doing. Here we are talking about a response which can be inhibited or stimulated The very stimuli which induce this response can also inhibit the response at other times It would be suggestive of magical thinking and induce much criticism of good observation to use language which induces criticism To say "block" as a result of injection of saline would give the so-called organicists who use a saline injection as a placebo to say, "Why, you have proven that you are wrong."

Fremont-Smith: Is not the question of suggestion important to try to evaluate here?

Mirsky· I wish Dr. Harrower would speak from her experience with testing I would like to know, for example, something about the subjects who demonstrate the responses in these areas; are they hysterical personalities? One gets the impression, for instance, in the hand-glove —

Travel That is a special situation.

Mirsky: Are we dealing there with a marked hysterical character who is very suggestible? I would like to know what happens when sprays are used and the patient is asleep

Fremont-Smith· You wake him up

Mirsky *The spray is not being used so that it* would wake him up

Fremont-Smith The intensity of the cold feeling, even though the skin may not be—

Mirsky. If the patient perceived the cold feeling—is it peripheral, or is it central? That would answer that question

Travell I am sure the spray would wake a person. It is very difficult to set up proper controls for a physical agent.

Holbrook I think this is closely associated with the problem which we faced a great many years ago in the description of fibrositis I am sure Dr Campbell will remember that many times we have tried to find out what the pathology of the so-called fibrositic nodule is, and every time we cut it out it is not there That is the amazing thing about this description of trigger mechanisms in which there are no evidences of inflammation nor of anything that can be determined as an abnormal

normal muscle, it does not Is that right?

Travell· It is correct that there is almost always a localized motor response on inserting the needle into a trigger area, but I am not sure it is a spasm A localized twitch is also often seen when the trigger area is palpatated.

Mirsky I would like to ask two questions When one trigger area is being eliminated, if one simultaneously injures another part, does one set up a new trigger area?

Travell You mean that if during the course of treatment of one region the patient sustains another injury, does this set up another pain syndrome somewhere else? It can and does happen at times It is not a universal phenomenon

Holbrook Dr Travell said that you cannot produce a permanent trigger area experimentally, you can only produce a transient one

Travell We have no technique for consistently producing a trigger area with a lasting pain cycle The only way I know is to activate a *latent* trigger area by injecting a local irritant, which may sometimes start a persistent pain cycle—but there is something wrong to start with

I would like to come back to the question of suggestion

Of course, it plays a role in any form of therapy and we are well aware of the importance of placebo effects (34) But suggestion is not the whole story here

In treating these trigger areas by local infiltration, you only get rid of the one that you inject Now if the effect is due to suggestion, you ought to get rid of a whole lot of trigger areas You ought really to clear up the syndrome, but you do not Furthermore, you can make inj

an

to

better or that he will be better tomorrow, but if you have not hit the trigger area and set off the pattern of pain, he will not be any better You have just failed to influence it, and I would say that that is the best evidence we have against suggestion as a primary factor

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Tremont-Sm [illegible]
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In treating these trigger areas by local infiltration, you only get rid of the one that you inject Now if the effect is due to suggestion, you ought to get rid of a whole lot of trigger areas You ought really to clear up the syndrome, but you do not Furthermore, you can make injections and go through all the motions and not hit the trigger area, and then, in spite of the greatest power of suggestion that you can bring to bear, you achieve no benefit You can assure the patient that he is better or that he will be better tomorrow, but if you have not hit the trigger area and set off the pattern of pain, he will not be any better You have just failed to influence it, and I would say that that is the best evidence we have against suggestion as a primary factor

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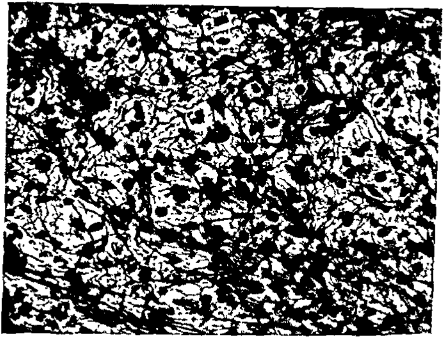


FIGURE 47 * Photomicrograph of part of a 72-hour-old culture of chick embryo skin fixed in Zenker-formal and stained with silver. Mag × 325

*It is a pleasure to acknowledge the collaboration of Dr Parker Vanamee in the preliminary studies which provided the microphotograph shown in Figures 47, 48, 49, and 62



[illegible]

H_2O and dried for microscopy The reference line represents 1 micron on this and the following figures Mag × 16,000

unit fibers because they have no apparent subdivisions, and because they persist as units in the parallel organization of the large strands It is also possible, I think, to see—although the magnification is not really adequate—a periodicity or a striation in these fibers (at C) which, according to its dimensions — 640 Å — describes the strands as collagen This characteristic periodicity is perhaps better shown in the next micrograph (Figure 49) which pictures a shadowed preparation of a similar sort of material.

The relation of these fibers to the cells, in the culture was difficult to see in these *preparations*, and the comment was published (15) that there was no evidence to indicate that the fibers were derived from the cells or spun off their surfaces

Dempsey You spoke of the small fibrils as the unit fibril, as if y



[illegible] to that shown

had never seen anything smaller than that Is that correct?

Porter I simply meant that within these mats of well-formed fibers, which represent the fiber arrays in cultures about eight days old, there is this type of fibril which predominates and is uniform in size, and is itself not compounded of other fibrils I have seen smaller fibrils, and intend to show you some of them.

Ragan: How wide are they?

Porter: Four to 600 Å Much finer fibrils can be seen in the background "A" of Figure 49.

At the time we made these initial observations, we were also studying the mode of fiber formation in fibrin clots (17), and had noted in that situation that the polymerization takes place in a series of steps First, very small fibrils (protofibrils) appear which then coalesce to

form larger ones, these in turn do the same to form finally the fibrin strand of the clot. It seemed to us initially that the collagen fibers developed in much the same way, and that actually the fine fibrils in the background ("A" in Figure 49) represented the earliest form of the fiber, and that these came together to form the unit fibrils, and so on, to produce finally compound strands

When, however, we examined the material a little more carefully, it was noted that unit and compound fibers in mats are not disposed at random, or woven like the fibers in cloth, but are put down in a laminated fashion. [See also (14).] If you examine the course of any of the strands in Figure 50, you will notice that they do not weave in and out of the mat "A" in Figure 50, for example, does not go over one, under the next, and over the following one, and so on In other words, in the dimension normal to the supporting surface, the fibers are arranged in planes above or below one another This suggested that fiber formation had *not* proceeded freely in all directions as in the formation of fibrin strands in clot development or as might be expected if the

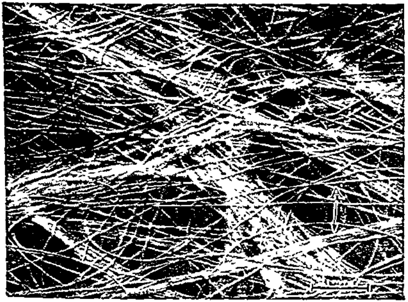


FIGURE 50 Mat of collagen fibers in 8-day-old culture of chick embryo skin Mag × 21,000

collagen fibers polymerized out of an intercellular matrix. It seemed rather that the fiber mat had been organized by cells, which, in migration, had deposited new fiber arrays above or below those already formed We were thus led to conclude that our earlier notions on the origin and development of collagen fibers were probably erroneous and that we had better examine the problem further

In doing this we chose a simpler culture medium and followed very closely the procedures Margaret Lewis had used in her much earlier studies of this problem (5). Her findings that the fibrils which appear in culture develop in the "exoplasm of the cell" have since been variously treated but generally not accepted because she provided no evidence that the fibers observed were reticulin or collagen They have been interpreted as fibroglial fibers [See (11).]

In our more recent experiments we have used cultures of skin, of eight- or nine-day-old chick embryos set up in a medium containing no



FIGURE 51 Low power photomicrograph of the margin of cells which grow and migrate from an explant of chick skin in Locke-bouillon medium during 24 hours of incubation A portion of the explant is at the lower lefthand corner Mag × 115

fibrin or serum components, nor embryo extract but only balanced salt solution (Locke's with 0.25 per cent dextrose) and beef bouillon (10 per cent).

In such cultures, the cells grow out in a thin sheet on the surface of the cover glass, as shown in Figure 51. They are thin and excellent for electron microscopy. In preparation for this they are simply freed of any tissue debris and the bouillon by rinsing in a fresh quantity of balanced salt solution and placed in the presence of osmium tetroxide vapors for fixation. Ten to twenty minutes suffice for this, and the fixed cells are thereafter transferred to the electron microscope grid and dried.

Figure 51 represents the growth which takes place in twenty-four hours. During the next twenty-four to forty-eight hours, the margin of cells increases as much again in terms of the width of the margin migrating cells. Then under these conditions of culturing, the growth generally stops. Presumably, the media and the conditions of culturing will not support any further expansion.

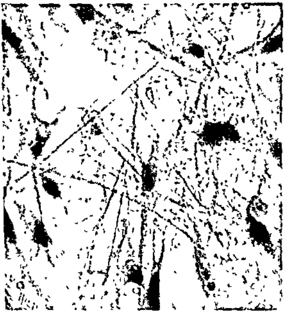


FIGURE 52. Higher power photomicrograph of chick skin culture after 6 days growth and differentiation in Locke-bouillon medium. Fixed in OsO_4 vapors and stained with hematoxylin and eosin. Mag × 450.

From this time on, for three or four days (longer if the medium is changed), the cells survive and in terms of fiber production, they differentiate That such preparations produce fibers is indicated in the next photomicrograph (Figure 52) of a portion of such a cell colony This is a hematoxylin stain preparation

The electron microscope naturally provides a more detailed picture of such fibers and shows that they are in fact collagen or have the structural characteristics of collagen fibers (See Figure 53.) These were in six-day-old cultures

You will notice that the striation is much finer actually than that of collagen I will come back to this later But there is a major period forming (at "A" in Figure 53) where each group of three fine striations is set off as a major group by an enlargement of one or two of



FIGURE 53 Electron micrograph of compound strands and unit fibers of collagen which developed during 6 days *in vitro* from explant of chick embryo skin Medium plasma-free, contained only Locke-bouillon mixture of Lewis (5). Mag × 30,000.

the three The point to be stressed here is that these, as near as we know anything about them, represent collagen, and therefore fibrin and serum components of which none was in the medium are not essential to collagen formation The fibers or the material to form them must have come from the cells of the culture

Now what is the origin of these fibers with respect to the cells? It is impossible to examine living cells with the electron microscope and watch fiber formation We were obliged, instead, to preserve representative cultures at twenty-four-hour intervals and reconstruct events from large collections of micrographs and observations In doing this, we were examining the sequences in the differentiation of embryonic cells which produce collagen The same sequences probably take place in tissue repair, or wound healing, where a primitive type of cell presumably becomes the active fibroblast

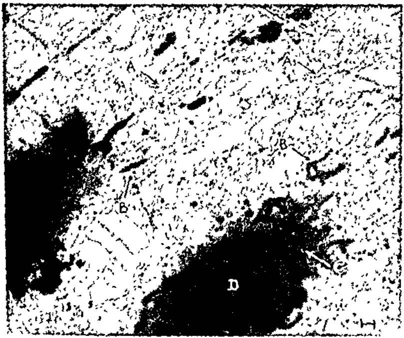


FIGURE 54 Electron micrograph of parts of two adjacent fibroblasts from chick embryo skin explant Culture 24 hours old Cells fixed in vapors of OsO_4 30 minutes "A" marks early fiber forms, "B," mitochondria, "C," centrosome, "D," nucleus Mag × 4,500

Fiber production increases with the age of the culture as would be expected After twenty-four hours, they show very little in the way of fibers There are a few strands at "A" in Figure 54 and this is what *is commonly found in these young, twenty-four-hour-old cultures After* seventy-two hours, the number of fibers associated with the cell has increased considerably (Figure 55). The kind of density along the cell margin ("A" in Figure 55) and sometimes occurring at numerous other points in the cytoplasm, is referred to as stress fibers or tonofibrils They are common in these cells.



FIGURE 55 Electron micrograph of fibers and cells in 72-hour old culture of chick embryo skin "A" refers to tonofibril or fibroglia fibers, "B," collagen fibers. Mag × 4,500.

After six days, or 144 hours in culture, the fiber collection is very rich (Figure 56). Actually, most of the ectoplasmic portion of the cell appears to be involved in fiber formation

Lansing: May I ask you a question? Perhaps I am not reading it clearly, but it seems to me there are differences between the surfaces of the cells shown. The earlier specimens seemed to show well-differentiated cell borders, almost a cell membrane of measurable thickness I think it was in the six-hour preparation Here it is almost entirely unrecognizable to me

Porter: That is right

Lansing: Did you attach significance to that transformation?

Porter: Yes, it is doubtless related to the observation we will make

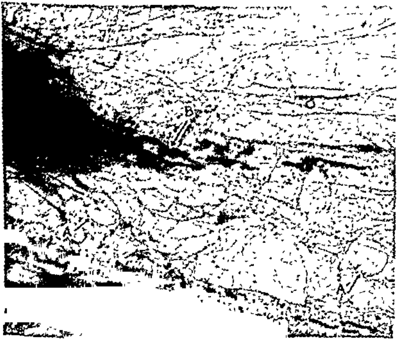


FIGURE 56 Electron micrograph of parts of 2 cells and fibers from 6-day old culture Mag × 4,500

Lansing: The implication almost is that the cell membrane is contributing to the formation of the fibers, to be consumed in so doing

Porter: I doubt that, but, nonetheless, it could be. One thing that is fairly striking here, and is common in these cells of older culture, is this

e at "A" in

"B" in Figure 56?

Porter: Yes, but this micrograph does not show the internal structure of the cell to good advantage.

Dempsey: May I ask a question for clarification? The fibers one can certainly see outside of the cell or near the cell, and in some cases in the same region as the cell. I do not believe you stated clearly whether they were actually within the cell, or lying superimposed upon the surface of the cell.

Porter. As I proceed your question will be answered

In early stages of fiber formation, or fiber production, it is possible in many cases to trace the fibers back to a cell, and usually to some region of its surface It is common to find them either developing in bundles, in long densities in the cell body—with the form and disposition of stress fibers (18) or fibroglial fibers (19)—or pulling off the surface as unit fibers and frequently as bundles of fibrils from unorganized fibrous looking material.

Figure 57 shows a portion of a cell margin "A" is the nucleus, "B" are mitocondria and "C" is the margin "D" would be referred to as a stress fiber or fibroglial fiber They are definitely a part of the cell body. At "E," which is the pseudopodium, you will notice that something is being pulled off the surface.

MacLeod. What do you mean, "being pulled off the surface"?

Porter. It is as though you have a mucinous material which pulls from a broad base out into a slender strand

Fremont-Smith: Similar to molasses

occur-

eous—

you can see it, whereas the outside space is made up of many holes, as it were It is a space

Porter You mean at "F" in Figure 57?

Hill: Yes

Porter No, "F" represents the substrate on which the cell is supported

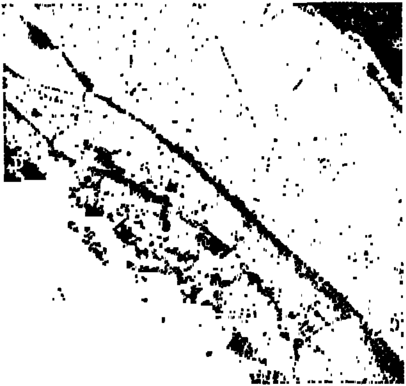


FIGURE 57 Micrograph of cell margin showing fibers and fibrous arrays apparently developing from surface of cell Taken from 72-hour-old culture Mag × 5,000.

Hill Is that the space in which there are fibrils?

Porter There is a film of plastic represented by "F," a supporting membrane It is not space These cells have to be supported on some thing for microscopy

Hill What does gelatin look like?

Porter It would look like the background—not like fibrils—as you see it at "F." These which cross the open spaces at "F" are extracellular, already formed

If these cell surfaces are examined under higher magnification, or formations of that type (at F) are examined under high magnification, we begin to see details as shown in Figure 58 You can see that very fine fibers are forming at the surface and bundling up, pulling

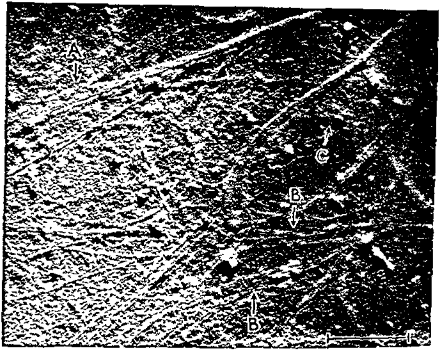


FIGURE 58 Portion of surface of cell from 6-day-old culture Shows extensive differentiation of polymerization of fibrils at or just within cell surface Mag × 30,000

into the strands, which finally find their way out of or off the cell I am not sure that it is detached yet, but it is certainly on the upper surface of the cell

Ragan Where is the nucleus in Figure 58?

Porter. I suspect it is toward the lower left corner

Bennett The fibroglial fiber—does it have periodicity?

Porter Yes, it appears to, but we will discuss that in a moment You see the same sort of parallel array is shown at "A" and at "B" At "C" can be seen one of these circular arrangements mentioned earlier. (See Figure 56)

It can be seen that some of the fine fibrils lose themselves in the material of the cell Presumably, they represent the earliest fibril of collagen Such, at any rate, is the present interpretation. I do not think there is much possibility of these fibrils having existed in the fluid over the cell and then being deposited extracellularly by drying, because they blend too convincingly with the material of the cell surface and furthermore, if loose they would have been washed away at the time of fixation



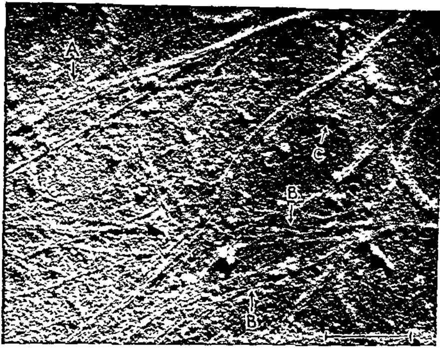


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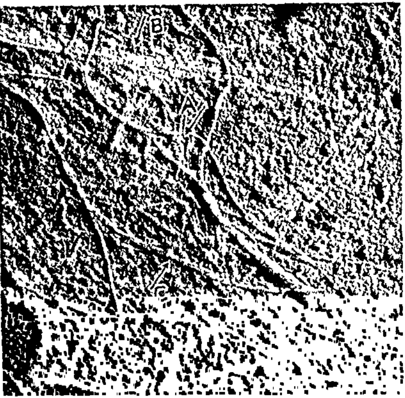


FIGURE 59 Another micrograph of cell surface to show intimate relation between cell fiber development Derived from 6-day-old culture Mag × 30,000

Figure 59 provides further evidence of this intimate relation in cell-fiber formation This micrograph perhaps has better resolution, you can see that the small fibrils are striated and have the micro periodicity (ca 210 Å) found in collagen One can note that a number of fine fibrils are twisting into this larger group at "A" Presumably, if another cell should pass over this one in any direction, or make contact with it, this would catch and be pulled off If this happens the fibers might be said to be spun off the surface

Gersh This is on the surface?

Porter Yes

MacLeod Are the granules cytoplasmic?

Porter: No, they are probably the macromolecular units which contribute to the fiber formation It is difficult to decide at these levels of minuteness

A parallel organization of the fibroglial type is developing at "B"

Fremont-Smith. Do you see any of these apparently deep within the cell, or are you suggesting that you see them primarily on the surface?

Porter: Wherever you obtain resolution of this fine periodicity (at "C"), the fibrils are doubtless on the surface If they were under the membrane or under any sort of layer material, that would probably not show

Zweifach In the microphotographs which you show, is there any appreciable depth of focus? Are we seeing surface of a cell or looking into its interior as well?

Porter. Actually, in this particular image, you have only a picture of the surface material because the shadowing enhances the contrast of all the surface structures The depth of focus in electron microscopy is 1 to 2 micra

Holbrook What you are really showing is a relief map

Porter. Yes

Gersh. Have you tried other fixatives?

Porter. Yes Formaldehyde, if properly used, gives a similar image.

Gersh. Are the granules of the same size?

Porter. Yes

Mirsky: What would you see if the fibers were covered?

Porter. You would see a little elevation in the surface of the cell. more or less smooth

Lansing. Is it your thought that the function of the fibroglial fiber is more or less—

Porter: My current feeling is that a group of fine unit fibers may form in or come to constitute a fibroglial strand, but this requires much more study.

Lansing. It does not contribute to the formation

Porter: It is the site of fiber formation

Ragan Where is the fibroglial fiber in Figure 59?

Porter: There is none very evident but "B" is probably the root or end of one

Ragan. That has no periodicity?

Porter I think you will see some if you look at it closely. This is deeper in the cell material than the fibril at "C" and not so completely

[illegible] polymerized

[illegible] oglia?

[illegible] of parallel fibers and their intracellular location

György: Why this distinction? Is it artificial?

Porter: The only excuse for applying these terms is to relate the observations to those made earlier with the light microscope.*

György: They are collagen fibers?

Porter: Yes, they apparently give rise to collagen fibers.

Fremont-Smith: Are you suggesting, or is it possible or reasonable to suggest, that the fiber formation then is an interphase phenomenon?

Porter: I suppose in a sense it is; yes.

Campbell: Do the fibroglial fibers seem to be laminar in relation to the surface of the cell?

Porter: I have not noticed.

Morrone: Are all those fibers collagen?

Porter: Some that pull off do not organize very well. It is difficult to decide about them.

György: The fibroglia never leaves the cell?

Porter: The collagen differentiates out of the surface, through the cell, and then will pull off of the cell as a bundle of collagen fibrils, unit fibers. Whether they are referred to as fibroglia fibers while still in the cell is not important. I think the main point that I can make convincingly is that the collagen fibrils do form at the surface of cells. Our studies have not been extensive enough to define definitely the fibroglial fiber and its relation to the ultimate collagen fiber.

As mentioned earlier, it is difficult to decide on the relation of the cell membrane to all this, but it is reasonable to propose that the ectoplasmic material—that is a plasmagel layer† just beneath the membrane, may organize into collagen fibrils while still beneath the cell membrane, or that this material may pass through the membrane as an exudate, and then, on the cell surface, polymerize into definite fibrils.

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*Fibroglia fibers were named and described by Mallory in 1902 (19) as a class of intracellular fibrils which appear red after the acid fuchsin-aniline blue collagen method. They coincide in distribution and form with fibrils described by Maximow at about the same time and called by him, tonofibrils (20). Mallory notes that "these fibrils follow closely the fate of the connective-tissue cells: they are numerous and prominent when the cells are abundant, active and proliferating; they are hard to find when the cells are few in number and in an inactive state." Thus they are common in cells in granulation tissue and at other sites where collagen is being deposited. He describes them as running through the outer surface of the protoplasm. They may, moreover, pass from cell to cell.

†The discussion which follows would have been improved if the term "plasmagel" had not been introduced. The ectoplasm is the cytoplasmic layer just beneath the cell membrane which is regularly free of cytoplasmic particulates and is usually in the gelled state. Other portions of the cytoplasmic ground substance as well as the ectoplasm undergo sol-gel transformations and, at appropriate times, are plasmagel or plasmasol. Ectoplasm is then the more restrictive term and seems the more likely site for fiber formation.

Presumably the motion of cell over cell, and over substrate, and the consequent stresses play a role in organizing fibers into broad bundles or arrays essential to the tissue to which they belong.

After leaving the cell as

of the order of 200 to 300 Å

appear to increase in diamet

may be of the size shown as "A" in Figure 60, but after they leave the cell, they grow to the dimensions shown in Figure 61, from 200 Å to something like 500 Å or 600 Å.

Lansing You referred to the plasmagel. Is that demonstrable in any



FIGURE 60 Small area of surface of cell from 72-hour-old culture grown in Locke-bouillon medium Only fibrils are developing out of cell surface Mag. × 30,000



FIGURE 61 Portion of fiber mat put down in embryo skin culture after 6 days Locke bouillon medium Mag × 30,000

of the electron micrographs? Is there a differentiated zone recognizable?

Porter No, not sharply differentiated It is a hyalin layer continuous, i e, diffusely continuous, with the endoplasmic interior of the cell I have no material with me to demonstrate it as it appears in these electron microscope images

Lansing I am very sympathetic to plasmagel However, I would like to see it

Porter It is demonstrable

Fremont-Smith It is removed in the living cell by micro—

Porter: It cannot be removed in the living cell. We remove it in the fixed cell by prolonged fixation with osmium tetroxide Apparently this fixation over several hours decomposes the material of the ectoplasm and it diffuses out

Fremont-Smith: If you suck out this material with a needle, does that leave membrane behind, a dried-out cell?

Porter. That has not been done to my knowledge It is probably not detachable from the under side of the membrane.

Lansing: The point is that we are differentiating cell membrane from plasmagel

Porter· There is a formed structure which is a cell membrane, and just beneath that there is a region, very thin, which is referred to as ectoplasm It is usually in the gel state and in such a state may be referred to as plasmagel

Dempsey: And those two components continue out in the pseudopod, as well as occurring—

Porter. The ectoplasm is everywhere under the surface and is not excluded from the pseudopodia In cell migration it exhibits sol-gel transformations

Fremont-Smith In the flow that one sees, that is the plasmagel.

Gyorgy. You mentioned that plasmagel may be extruded.

Porter. In the case of fibroblasts or fiber-forming cells, it is conceivable to me that it could come through As a matter of fact, from our experience with these fibroblasts, I have come to wonder whether they have a membrane as well defined as that on epithelial or endothelial cells

Lansing Do I have the right idea, that it is your thought that the plasmagel contributes to the chemical make-up of the fiber?*

Porter I think that the collagen elements, the molecular elements which go into collagen formation, are probably in the plasmagel (ectoplasm) before they form fibers, and may actually be polymerized into fibers at that site of the cell structure

Lansing· Would it follow that the chemistry of the fiber then would reflect the chemistry of the plasmagel?

Porter· Yes, it would.

Lansing: Let us say that the amino acid analysis of collagen would be the amino acid analysis of plasmagel Is that going too far?

Porter. Not necessarily.

Angevine· Is there any relation between the number of pseudopods, and the age of the fibroblasts, or to the number of collagen fibrils that are formed?

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*See Footnote on Page 143

Porter. The only relation I know of is one to the age of the cell *in vitro.* What we are actually doing, if it is a six-day-old culture, is observing the collagen production of a fibroblast in a fourteen-day-old embryo which, in the skin of the fourteen-day-old embryo, is surrounded by plenty of collagen

Lansing One more question on the plasmagel Is the plasmagel that you refer to comparable to the plasmagel used by Heilbrunn and his students?

Porter Yes

Lansing It is the same plasmagel

Porter I believe so All of the experimental cytologists—

Lansing I want to make sure of my terminology It is a classical plasmagel that we are talking about here

Porter Now, the mode of increase in diameter of these fibers has not been evident, but I think it is reasonable to assume that a collagenous component, or a component of the fiber, is deposited from the media on the fiber, after it is detached from the cell surface

In other words, there is an accretion of collagen, or a condensation of collagen after the fiber leaves the cell, and the fiber thereby increases or grows in size Growing suggests that the fiber has much to do with it

Hill Where does the collagen come from?

Porter If there is collagen on the surface of the cell that can form fibrils at the surface, it is reasonable that some of it would go into solution without polymerizing, and then from the medium—

Hill Which medium?

Porter Around the fibers (the culture medium), outside the cell, extracellular—it might condense out on the previously formed fibrils, i e , the fine ones, the protofibrils

Dempsey In other words, the fiber could grow by apposition The organization or fundamental periodicity of the fiber may be laid down by the cell, from that point it is a matter of plastering on more units

Porter The interesting thing to note in this is that collagen fibrils of this type—unit fibers of different tissues—are of different diameter, but *among themselves are almost uniform It is just as though the skeletal* fiber which is put down by the cell is capable of taking on only a certain thickness of all this material on the outside and holding it

MacLeod You would say an adding on, as opposed to swelling of the fiber

Porter I frankly had not considered the possibility of swelling If it swells, it has to do so by taking up nonvolatile material because these fibers are all dried and even so have dimensions which vary with age

A number of interesting phenomena come to light at these levels of resolution In mentioning them I should point out that they need more study for proper definition For example, along with this increase in fiber diameter, there appears to be a change in the striation pattern The fibers, shown in Figure 62, are recognized as more or less mature, in that they have the form found in adult tissue (at "A"). That is, two striae somewhat fused are separated by a depression .



FIGURE 62 Collagen fibers of the adult type developed in explant of skin of 12-day-old embryo after 8 days in medium containing plasma and embryonic extract Mag $\times$ 33,000

In the young fibril, as marked by its diameter and cell relationships this same distance or space, 640 Å, is occupied by three striae of uniform, or nearly uniform, size. You can see (Not illustrated, but the structure is the same as in fibers shown in Figures 53, 60, or 61) that *the striations are nearly all of uniform size. It takes three of those to* make a 640 Å period. In other words, these are about 210 to 220 Å. It is interesting and perhaps of some significance that fibrin has about the same periodicity.

György: What is the difference between the two?

Porter: The striations are uniform.

György: What is the difference in age?

Porter: The collagen fibril with the uniform striae is younger by several days.

Another observation is that in Locke-bouillon medium, we have not found mature, completely-formed collagen of the type shown in Figure 62. This was grown in the presence of plasma, whereas the fibers in Figures 53 and 61 were formed in Locke-bouillon alone. There is enough evidence in this latter material to show that occasionally (as at "A" in Figure 53) every third of these striae will expand in size to give the 640 Å periodicity. When this differentiation goes further every second and third striation adds on material and the third one disappears into the depression between the major bundlings (Figure 62).

György: You do not find the same difference in what you call reticulum fibers and collagen.

Porter: I really cannot make any distinction.

György: They are always the mature type?

Porter: Yes. Another thing frequently noticed in these cultures is the presence of an interfiber material not generally fibrous, but put down in a metameric pattern, as shown at "A" in Figure 63. The periodicity of that, is not 640 Å but 1100 Å, nearly twice that of collagen.

Dempsey: Is it free?

Porter: It is in between the collagen fibers.

Dempsey: Extracellular?

Porter: Yes. It fills in between them.

Travell: Where does that come from?

Porter: It must come from the cells in the skin culture.

In the absence of collagen fibers, it may form spindle shaped units showing precise periodicity.* Tentatively, we interpret this as representing the ground substance that Dr. Gersh describes. In other words, possibly a muco-protein. When Dr. Gersh speaks of polymerization of ground substance, he may be referring to the deposition of this material *in this pattern of organization.*

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* These observations made in collaboration with Dr. Parker Vanamee will be published in detail elsewhere. (20)



FIGURE 63 Portion of culture of chick embryo skin showing large-period, interfibrillar material Medium, Locke-bouillon Age of culture 6 days Mag × 18,000

Actually, if you look carefully at Figure 63, you will see that in this particular portion of the fiber array, the collagen strand at "A" has developed the adult type of periodicity, whereas most of the collagen shown here is not that well formed There is then a suggestion that this larger period material is continuous with the collagen fiber and that it may have contributed to the development of the adult type periodicity.

Now, the particular technique we are employing, i e, culturing in a well-defined medium, provides fair possibilities for the investigation of these and other problems, since a variety of environmental conditions may be controlled and changed to suit the purposes of the study.

For example, effects of varying the culture media may be readily noted Leaving out the bouillon from the culture makes no essential difference in the production of fibers. The cells do not grow quite as well but collagen fibers of the same type are produced

Adding a portion of whole serum, to the medium, on the other hand, leads to a pronounced increase in fiber formation It is probably a nutritional effect.

Figure 64 for example, shows a portion of a culture grown in Locke-bouillon without protein added Figure 65, on the other hand, is of parts of cells grown in a medium containing serum (25 per cent by volume) You will notice that the fibers are more abundant in the latter.

Mirsky: If you had a different type of medium, would you get the same thing?

Porter: I have not varied the medium, except for one experiment

I anticipated that someone in this group would ask what cortisone-like compounds might do So we set up a series of cultures, one-half of which received 5 gamma per ml of compound F acetate The colony size and the cell migration were essentially the same in both cultures, but the fiber production was quite different In the cultures without

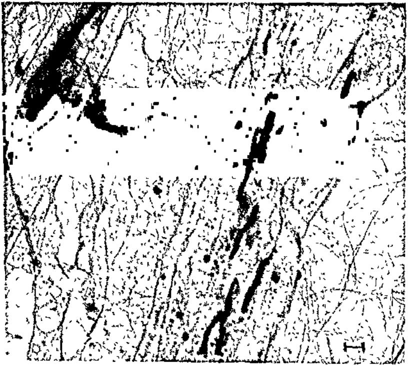


FIGURE 64 Parts of 2 cells with surrounding collagen fibrils grown in Locke bouillon medium Mag × 6,000



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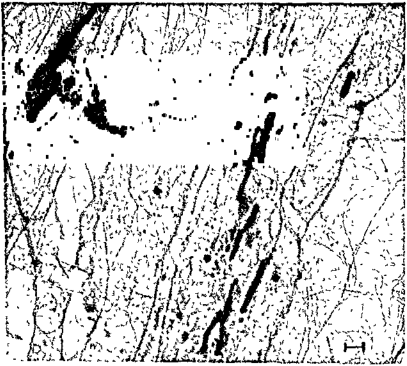


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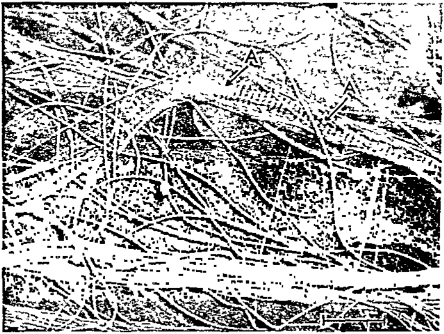


FIGURE 63 Portıon of culture of chıck embryo skın showıng large-perıod, ınterfibrıllar materıal Medium, Locke-bouıllon. Age of culture 6 days Mag × 18,000.

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large [illegible]
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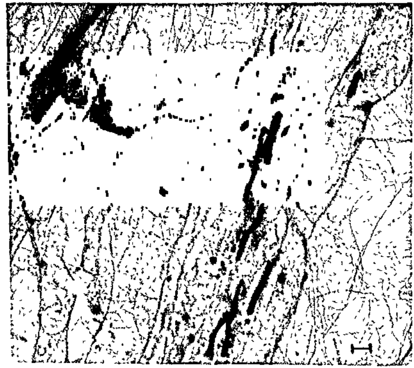


FIGURE 64 Parts of 2 cells with surrounding collagen fibrils grown in Locke bouillon medium Mag × 6,000

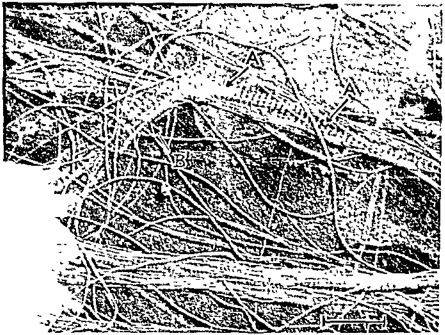


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FIGURE 64 Parts of 2 cells with surrounding collagen fibrils grown in Locke bouillon medium Mag × 6,000

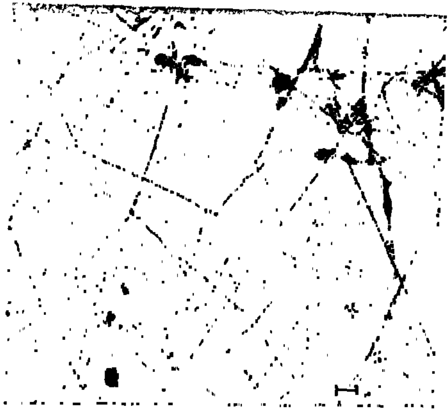


FIGURE 65 Parts of cells which were cultured simultaneously with those shown in Figure 64 in medium containing 25% chick serum in Locke-bouillon Mag × 6,000

compound F, collagen was present at the end of seventy-two hours as normally, but in cultures with compound F, there was less collagen In fact, in some colonies, we could find no evidence of collagen production whatsoever Where fibers were present, I suspect they might have been drawn from the explant by the cells as they migrated.* This observation is, I believe, in agreement with other observations in animals, made on *in vivo* development of collagen as e g in the suppression of granulation tissue formation (21).

Zweifach: In tissue culture, is fibril formation seen only in association with fibroblasts? Do any of the other cellular elements show fiber formation on their surfaces? It is not clear to me whether the deposition of this material is exclusive with the fibroblasts or whether it

*Subsequent repetitions of this experiment have not given consistently striking differences possibly because compound F has not gone into solution to the same extent in each case.

occurs indiscriminately with all of the cellular elements undergoing active growth

Porter: It is difficult to say that such and such a cell is a fibroblast in culture. I speak of these as fibroblasts because they are derived from the dermis and subcutaneous portions of the skin of the embryo, where the population of fibroblasts is relatively high.

Zweifach: Many of the cells in tissue culture preparation show active ameboid type movement, with pseudopod-like structures As these cells extrude and withdraw their pseudopodia, do they leave behind a fibrillar matrix or structure?

Hill: I am trying to correlate these results with our work, because, let me repeat again, we have an eosinophilic coagulum which so far gives all the tinctorial reactions of collagen The only correlation I can see is a statement that once the fibers are outside the cell, they perhaps enlarge by creation of a protein substance, possibly collagen protein, which literally floats about from the cell to the fiber, the core of which is already there The question is, is that what we have?

Porter You said yesterday that your coagulum was acellular in your experience and it occurred to me, of course, that there may have been cells in there at one time, it would not take them very long to disappear, would it?

I do not exclude the possibility that collagen polymerizes outside of the cell In fact, if it can polymerize from the material on the surface of the cell, I do not see why, provided the concentration of that same material were high enough elsewhere, it should not polymerize into fibrils The important thing, I think, is that the cell can apparently function in this manner, that the fibers do polymerize at or on the surface, and that gives the cell the opportunity to organize collagen fibers as they need to be organized for the particular tissue

Hill. This periodicity that you show in your fibers—do you think that is a vibratory effect?

Porter That is an interesting question It brings to mind what we have seen in these small fusiform fibers with the large periodicity, this supposed component of the ground substance Sometimes at the ends of them, where they taper off, there will be a striation, a tiny pile of material, and then a space, and no connection visible between this and successive striae In other words, it almost seems as though there is a field effect, which is orienting the terminal striae beyond the limits of the fibers Long-range forces are in doubtful favor these days, but *possibly these results are mediated through some vibrator means There* is also something curious in the collagen fiber itself which in the growth of the fiber causes material to be added at only two of every striae instead of all three

Hill: Would it have an effect if you had a supersonic device?

Porter To see if you could interfere with the organization of forces, which we are supposing exist around the fibers? It might be interesting to try it

Hartroft. Could your concept of additional extracellular growth of the fibers be applied to the question of the transformation of reticulin fibers to collagen fibers? In pathological conditions is it possible that such a transformation represents an abnormal extension of what you have just illustrated? Or on the other hand are collagen fibers formed by ordinary reticulin fibers of average size which have increased in number and finally coalesced into larger bundles?

Porter. The latter has been my concept There may even be a matrix in such large bundles which changes the staining properties I mean that something fills in between the reticulum fibers, perhaps the same material that contributes to the development of the adult type of fibril.

Hartroft: Does this represent a relatively adult fiber?

Porter· Yes, from an older culture

Ragan: If you fix with formalin instead of osmium, do you notice any difference in interfibrillar material?

Porter· I have not done enough with formalin to answer that

Hartroft. Have you ever tried to correlate the appearance that you have shown with the standard staining procedures as observed with the standard microscope? Would the material in Figure 65 give the commonly-accepted staining reactions of collagen?

Porter. The silver technique could be applied to these materials and it would be interesting to see by electron microscopy the relation of the silver particle to the unit fiber of collagen and the bundle. Unfortunately I have not done it The other stains for collagen would probably not show at all in the electron microscope

Hartroft· I realize the other stains would not be apparent with the electron microscope. But could you not stain a similar explant of the same age with the classical collagen stains (aniline blue, etc.) and examine it with the standard microscope?

Porter. The spaces would not be big enough to be resolved

Morrione You have not observed the effect of collagenase on collagen with electron microscopy? I was thinking of enzymatic experiments similar to those which Dr Lansing showed.

Porter· No It is certainly an ideal thing to do.

Lansing· Would you care to comment on the origin of elastic fibers?

Porter: I would rather not, because I really do not know anything about it

Holbrook Some of us got the impression from some of those illus-

trations, Dr Porter, that there appeared to be a tendency to coil. Is that so, or are these fibrils parallel?

Porter. Well, there may be a slight coiling Sometimes when these collagen fibers, especially in collagen derived from rat-tail tendon, are placed in dilute acetic acid, they swell, and the electron microscope image of the expanded or swollen fiber will show a very oblique coiling in its structure when it breaks down But I do not think there is much evidence of a similar coiling in this skin collagen

Ragan In reprecipitating rat-tail tendon, have you ever seen any fibrils with a periodicity of 210 Å?

Porter Yes, the small fibrils of reprecipitated material show it as do also the large ones (20)

Ragan At the proper pH?

Porter Yes, and under conditions of sodium chloride precipitation

Mirsky Would you comment on your impressions as to the mechanism of the steroid effect?

Porter It was ill advised even for me to mention it Having done so, I will tell you I read a review by Ingle (22), where the comment was made that it interfered with protein synthesis

Mirsky Did you find, when you used serum, or used the protein rich nutrient—that you obtained a similar picture?

Porter The dissimilarity in protein-free and protein-rich medium is one of abundance of fibers The fibers are there in the protein-free medium, but in lesser numbers The whole cytoplasm of the cell is less dense presumably less rich in protein But in cells treated with compound F, I did not notice any diminution in the density of the cells They simply had not formed collagen

Mirsky That is the reason I bring this point up If it were a question of an inhibition or distortion of protein synthesis, you would expect that the cell itself would show it But here you show almost a specific effect on fibril formation, rather than on the total protein structure of the cell

Porter Compound F does peculiar things to the cells I was amused to see its effect on the mode of migration The cells appear to have a lower viscosity They migrate, but the nucleus may pull way out behind as a long trailer

Ragan You mean they migrate less

Porter I wouldn't say so, but I have thought that the general intracellular fiber organization and production might be affected

I should mention that Dr. David Karnofsky gave me the compound F some months ago and urged me to try it at a time when he was studying growth-inhibiting effects of cortisone and compound F on chick

embryos (23). As you may know the effects he observed were not initiated until the eighth day of incubation, which curiously is just about the time that collagen starts to form (5).

Dempsey: Karnofsky obtained a considerable amount of growth, up to the eighth day, without any influence

Hill In your photographs, where does the extracellular matrix come from?

Porter Well, the picture is this *In vitro*, the culture medium surrounds the cells, but is added by us, and not by the cells. Anything else in the nature of a matrix is produced by the cells

Hill You show these extracellular fibers—you remember I asked about the holes There is a matrix.

Porter The spaces represent the substrate, not the matrix That is the surface on which the material is growing

Hill. The nutrient medium?

Porter No; this material is just supporting the cells You see, we take a cover glass, coat it with a resin film (Formvar), and then coat that with a monomolecular layer of albumin for the reason that the cells seem to migrate better on a protein-coated surface After the cells have grown out, we peel off that membrane with the cells on it and put it on the electron microscope grid What you see between the cells is the Formvar membrane and the absorbed protein It is not thick, only a matter of about 20 millimicrons

Campbell Is your culture forming ground substance too?

Porter Probably, yes

Ragan Is it washed out?

Porter. It is either washed out, or it goes into solution in the balanced salt solution or nutrient It is probably also washed out when we rinse the preparations before fixation

Ragan One possible explanation for your effect of compound F was that the cells did not mature to the point where they would form collagen, since you are dealing with mesenchymal cells

Porter: We have not reached the place where we are making such distinctions I can distinguish between a macrophage and a fibroblast.

Mirsky How?

Porter: By the round shape of the macrophage versus the spindle shape of the fibroblast. Macrophages also frequently show a prominent centrosome and ruffled pseudopodia

Ragan Does a macrophage ever have fibroglial fibers?

Porter I would say so A macrophage, if migrating, may show intracellular fiber arrays, condensations within the cytoplasm, but they are transitory. They form and disappear, polymerize and depolymerize all the time, as the cell moves

Ragan: I would like to ask one question, and that is, does this group believe that at least one of the stem cells of the fibroblast is the macrophage? Dr Gersh evidently implied yesterday that macrophages develop into fibroblasts You have implied it now There is still a great deal of disagreement among histologists How about you, Dr Dempsey?

Dempsey. I have no thesis to defend on that I would say that embryologically they all come from the mesenchymal cell I try not to get into arguments about the possible transformations of the adult forms

Porter It is quite certain that fibroblasts or fibroblast-like cells will transform into macrophages

Ragan Many people will say no

Porter They have not done much tissue culture work, we see it all the time

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REGRESSION OF SCAR TISSUE

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IN THE COURSE of studies on cirrhosis of the liver, we have been struck with the fact that, at least in the peculiar instance of experimental cirrhosis, we have evidence which leads us to believe that connective tissue can regress From my observations this regression is an elusive change and the tissue actually disappears

Deposition of fibrous tissue which occurs in cirrhosis as a result of the administration of carbon tetrachloride to rats is shown in Figure 66 This was administered to a group of 109 rats by inhalation, with exposure for three and three-quarter months, quantitative determinations of collagen upon samples of the liver were made, and the data were tabulated

In this treated group the average value for collagen was 1 01 per cent as compared to a group of normals which was 0 23 per cent (Table V) According to the chemical data, about a fourfold increase in connective tissue occurred in the group of twenty-five animals

We selected these animals at random from a larger pool, stopped the administration of the hepatoxic agent to the remainder for a period of one month, and then observed the reversal

I have calculated what we shall call total hepatic collagen, a figure arrived at by a multiplication of the per cent of collagen by the liver weight I thought that figure might be interesting to follow with the percentage changes, because as you will see, there is considerable vari-

seen that with reticulum stains, one can judge the amount of material which is determined by the Lowry method quite accurately (1)

Figure 67 shows an accumulation of material producing this pseudolobulation My impression is that in these experimental cirrhoses there is far more reticulum than collagen Certainly, in this Figure it can be

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We selected these animals at random from a larger pool, stopped the administration of the hepatoxic agent to the remainder for a period of one month, and then observed the reversal

I have calculated what we shall call total hepatic collagen, a figure arrived at by a multiplication of the per cent of collagen by the liver weight I thought that figure might be interesting to follow with the percentage changes, because as you will see, there is considerable variation in the weight of the livers in these animals

We also have tabulated the microscopic evaluation of collagen based upon reticulum stain sections, as in my opinion collagen and reticulum are the same substance The plus system is used, and later it will be seen that with reticulum stains, one can judge the amount of material which is determined by the Lowry method quite accurately (1)

Figure 67 shows an accumulation of material producing this pseudolobulation My impression is that in these experimental cirrhoses there is far more reticulum than collagen Certainly, in this Figure it can be



FIGURE 66 Cirrhosis in a rat following 3¾ months of exposure to carbon tetrachloride vapors Wide fibrous septae separate pseudolobules of hepatic cells Collagen content 1.10 per cent Histological grading of cirrhosis ++++ × 300 Reprinted by permission from Morrione, T G *J Exper Med* **85**, *217 (1947)*.

TABLE V*

Cirrhosis Due to Carbon Tetrachloride

Rat No	Rat weight	Liver weight	Collagen wet weight	Total hepatic collagen	Microscopic evaluation of collagen, based upon reticulum-stained sections
	gm	gm	per cent		
1			1 14		
2			1 27		
3			1 36		
4			0 89		
5	112 0	2 6	1 10	28 4	++++
6	139 8	5 2	1 32	69 2	++++
7	154 6	5 0	1 09	54 5	++++
8	158 1	9 1	1 83	166 5	++++
9	161 2	7 9	0 73	57 7	++
10	161 7	8 5	0 97	82 5	++++
11	163 5	8 4	1 82	152 9	++++
12	167 5	7 8	0 64	49 9	++++
13	172 6	8 9	0 77	69 1	++++
14	176 6	8 9	0 99	88 1	+++
15	182 7	10 2	0 73	74 5	+++
16	183 3	8 8	0 99	87 1	++
17	187 4	11 5	0 59	67 9	+++
18	197 5	9 6	1 48	111 1	++++
19	200 1	9 8	1 23	120 5	+
20	200 9	9 1	1 44	131 0	+++
21	208 2	7 8	0 57	44 5	++
22	209 8	10 0	0 79	78 9	+++
23	210 0	10 1	1 15	116 2	++++
24	227 4	9 6	0 89	85 4	+++
25	237 8	11 0	0 44	48 4	+
Average	181 6	8 6	1 01†	86 4	

†Standard deviation, 0 14

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FIGURE 67 Pseudolobulation in cirrhosis following exposure to car- [illegible] nounts of [illegible] Gomori [illegible] l grading ++++ × 300 Reprinted by permission from Morrione, T. G: *J Exper Med* 85, 217 (1947).

seen a good deal is formed This animal, for example, had a value of approximately 1 32 per cent, considerably higher, as you can see, from the 0 23 per cent, which would be normal.

In an ordinary black and white photograph of an H and E preparation, when we state that a cirrhosis is present, we may actually be reading something into it which we do not observe. In such a section some material is present which we assume is connective tissue, but one cannot see it

In a colored photograph we do see the color view of it, and therefore assume there is some connective tissue present. This is about the level

at which most pathologists work. The increase in connective tissue which has occurred in such a liver would not be well shown, but a silver stain would show many reticular fibers—connective tissue fibers—which are not seen in the usual connective tissue stains.

The bluish stained material seen in a trichrome stain is mentioned as an example only to say that it is far over-rated as a connective tissue stain. In other words, the first thing which we look for in our studies is a correlation between the microscopic observation of cirrhosis and our chemical data. When we tried to do that with the stain recommended for collagen, we were unable to do so.

Table VI represents a group of animals left alive from that original first pool without carbon tetrachloride. We notice that after a month the value falls to 0.69 per cent as compared to 1.01 per cent, which represents a significant decrease in the connective tissue content of these livers. Microscopically, there still is a great deal of cirrhosis.

Fremont-Smith: The weights have increased, have they not?

Morrione: These animals did better, at least nutritionally, after the

TABLE VI*

Reversal Group A
Carbon Tetrachloride Discontinued for One Month

Rat No	Rat weight	Liver weight	Collagen wet weight	Total hepatic collagen	Microscopic evaluation of collagen based upon reticulum-stained sections
	gm	gm	per cent		
26	181.7	8.7	0.67	58.3	+++
27	199.7	8.6	0.69	59.3	++
28	206.5	7.8	0.64	49.9	+++
29	212.9	10.1	0.86	86.9	+++
30	216.0	8.8	0.61	53.7	+++
31	223.9	10.9	0.86	93.7	++++
32	246.5	10.6	0.57	61.2	++
33	280.5	10.9	0.49	53.4	+++
34	280.7	14.1	0.99	139.6	++++
35	296.2	13.5	0.58	78.3	+++
Average	234.5	10.4	0.69†	73.7	

†Standard deviation, 0.17

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carbon tetrachloride was discontinued They ate better and they did gain weight

Fremont-Smith: Is not your figure for total much more significant than your percentage, because your percentage is partly compensated for by the increase in weight?

Morrione: Yes, that is true, although both values parallel one another very closely

Table VII shows that after two months, the decrease in the group of twelve animals, which were sacrificed, was 0 61 per cent. This is not statistically significant from the one month figure of 0 69 per cent.

In Table VIII the three-month group, however, does show a drop to 0 51 per cent, which is statistically different from the 0 69 per cent, showing that during the period between one and three months, there was a progressive decrease in collagen

It can be seen that we are beginning to get one and two pluses in our microscopic evaluation

TABLE VII*

Reversal Group B
Carbon Tetrachloride Discontinued for Two Months

Rat No	Rat weight	Liver weight	Collagen wet weight	Total hepatic collagen	Microscopic evaluation of collagen, based upon reticulum-stained sections
	gm	gm	per cent		
36	153 5	6 5	0 61	39 7	++
37	155 7	7 9	0 84	66 4	++++
38	186 3	9 4	0 57	53 6	+++
39	201 3	8 6	0 68	58 5	+++
40	210 0	8 3	0 54	44 8	+++
41	227 0	10 0	0 68	68 0	+++
42	227 3	11 1	0 33	36 6	++
43	227 5	10 1	0 30	30 3	+
44	240 5	10 8	0 86	92 9	+++
45	263 2	11 0	0 56	61 6	+++
46	293 6	11 5	0 67	77.1	+++
47	299 8	13 5	0 64	86 4	+++
Average	223 8	9 9	0 61†	59 6	

†Standard deviation, 0 15
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TABLE VIII*

Reversal Group C

Carbon Tetrachloride Discontinued for Three Months

Rat No	Rat weight	Liver weight	Collagen wet weight	Total hepatic collagen	Microscopic evaluation of collagen, based upon reticulum-stained sections
	gm	gm	per cent		
48	225 1	9 6	0 55	52 8	+++
49	231 4	12 6	0 42	52 9	++
50	244 4	11 4	0 39	44 5	+
51	257 9	13 4	0 50	67 0	++
52	273 8	11 5	0 49	56 4	++
53	285 7	13 3	0 49	65 2	++
54	301 6	14 3	0 59	81 4	+++
55	303 1	12 9	0 52	67 1	+++
56	309 5	12 8	0 60	76 8	+++
Average	270 7	12 4	0 51†	63 0	

†Standard deviation 0 01

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Hartroft Were all these animals on a normal stock diet at this time?

Morrione In this group

Figure 68 shows one of these animals at the end of the experiment, that is, following a three-month period of reversal of cirrhosis, showing that there still is considerable reticulum between liver cells

Cirrhosis was produced in a group of thirty-eight rats with *p*-dimethylaminoazobenzene Twenty-three of these were sacrificed at the end of five and one-half months, and a rise in collagen wet weight roughly double that of the normal, with a total increase in collagen of about twice per average liver was observed (Table IX)

This agent, as you know, produces various types of tumor, and although animals with large bulky primary hepatic carcinomas were seen, the samples were selected from sites in the livers removed from the tumors In this type of cirrhosis the septi are much more cellular, as a rule, than in carbon tetrachloride cirrhosis There is relatively less connective tissue which is reflected in the lower chemical values which were obtained



FIGURE 68 Scant residual argyrophilic reticulum following partial reversal of cirrhosis due to carbon tetrachloride Gomori reticulum stain Collagen content 0 39 per cent Histological grading + × 300 Reprinted by permission from Morrione, T G : *J. Exper Med.* 85, 217 (1947)

TABLE IX§

Cirrhosis Due to *p*-Dimethylaminoazobenzene

Rat No	Rat weight	Liver weight	Collagen wet weight	Total hepatic collagen	Microscopic evaluation of collagen, based upon reticulum-stained sections
	gm	gm	per cent		
1			0 73		
2			0 33		
3	87	3 1	0 49	15 1	++
4	112 7	5 3	0 61	32 0	++++
5	118 4	4 9	0 71	35 1	++++
6	127 1	7 3	0 35	25 5	+
7	139 5	7 9	0 50	39 9	++++
8	149 6	8 9	0 51	45 1	+++
9	151 6	9 5	0 46	43 5	++
10	159 4	13 0	0 56	72 9	+++
11	161 0	8 3	0 28	23 4	++
12	183 4	7 5	0 43	32 2	+
13	184 4	10 0	0 49	49 1	+++
14	191 8	33 6*	0 48	161 3	+++
15	193 3	9 4	0 43	40 3	++
16	198 1	11 3	0 31	35 0	+++
17	204 5	9 4	0 47	44 0	+++
18	217 4	9 7	0 34	33 0	++
19	218 0	10 1	0 85	85 5	++++
20	219 0	10 6	0 44	46 5	++
21	238 7	11 1	0 42	46 6	+++
22	244 0	7 9	0 34	27 0	++
23	281 4	13 2	0 49	61 4	+++
Average	180 1	10 1	0 48†	47 5	
Reversal of cirrhosis group On normal‡ diet for 1½ months following cessation of *p*-dimethylaminoazobenzene rice diet					
24	213 0	10 6	0 24	25 4	+
25	215 8	9 0	0 13	11 7	0
26	216 5	8 9	0 28	24 9	+
27	218 6	10 6	0 14	14 8	0
28	229 0	10 0	0 24	24 0	+

*Large bulky primary hepatic carcinoma

†Standard deviation equals 0 13 (animals 1 to 23)

‡Dog chow pellets

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FIGURE 69 Abundant collagen and reticulum in a fibrous septum in cirrhosis due to *p*-dimethylaminoazobenzene Gomori reticulum stain Collagen content 0 47 per cent Histological grading +++. × 300 Reprinted by permission from Morrione T. G *J Exper. Med* 85, *217 (1947)*

Figure 69 illustrates reticulum staining of a rat showing an increase in reticulum in cirrhosis due to *p*-dimethylaminoazobenzene, which is quite marked

Animals from the group treated for five and one-half months with *p*-dimethylaminoazobenzene were then put on a normal diet for one and one-half months and the values for collagen fell to a normal level. It seems as though almost all of the connective tissue which had been

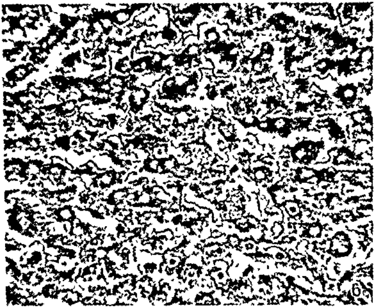


FIGURE 70 Sparse argyrophilic reticulum in recovery from cirrhosis due to *p*-dimethylaminoazobene Gomori reticulum stain Collagen content 0 24 per cent Histological grading + × 300 Reprinted by permission from Morrione, T G *J Exper Med 85, 217 (1947)*

laid down during the five months on *p*-dimethylaminoazobenzene disappeared quite rapidly and drastically within one and one-half months Microscopically, we saw no evidence of increased reticulum in some, and only slight in others

In Figure 70 can be seen a representative microscopic section from the liver of a rat from the original group which was put on a normal diet This shows just a sparse fiber here and there which would be a normal value chemically

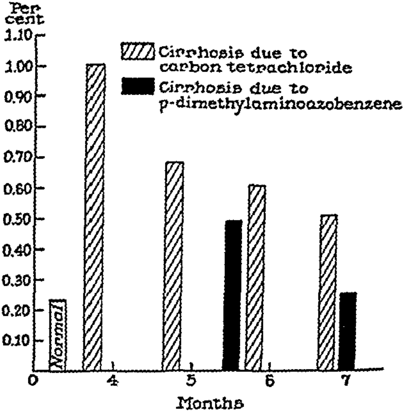


FIGURE 71 Graphic representation of alterations in collagen content in reversal of experimental cirrhosis Reprinted by permission from Morrione, T G. *J Exper Med* 85, 217 (1947)

TABLE X*

Correlation between Chemical Determination and Microscopic Evaluation of Collagen Content by the Reticulum Stain†

	++++	+++	++	+	0
Cirrhosis due to butter yellow	0 67	0 47	0 40	0 29	0 25
Cirrhosis due to carbon tetrachloride	1 14	0 68	0 58	0 59	

†*Numerical values expressed per cent collagen wet weight*
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Figure 71 graphically represents what we have shown so far

Table X gives the comparison between chemical findings and reticulum stain We took a series of about five hundred slides, some without cirrhosis and some with varying degrees of cirrhosis, and put them into five separate piles, according to the degrees of pluses We then looked up the chemical value on each slide, and surprisingly enough, with the reticulum stain, we were able to arrange the groups in a decreasing order of magnitude One cannot predict, on the basis of examination of cirrhotic livers with trichrome stains, what the chemical value is going to be But one can do quite well, I believe, with the reticulum stain We therefore assume that this method determines reticulum as well as collagen

Figure 72 illustrates graphically a more acute experiment in which we produced cirrhosis by administering carbon tetrachloride for thirty-five days We suspected that the original incomplete reversal observed was due to the fact that the cirrhosis was of prolonged duration Cameron (2) has shown that the duration of the cirrhotic state influences the reversibility and, presumably, the shorter the duration, the greater the likelihood of reversibility As you recall, we had a three and three-quarter month period of exposure to carbon tetrachloride in the first group of experiments In this group we produced a more rapid type of cirrhosis in only thirty-five days' duration by exposing the animals to the vapors In a group of 234 animals, in which we produced cirrhosis by this thirty-five day period of exposure, we sacrificed animals at five-day periods, so that each point shown in Figure 72 represents a group of at least five animals

I am no expert at analyzing curves, but it seemed to me that the sl between twenty-five to thirty days of carbon tetrachloride is more ac

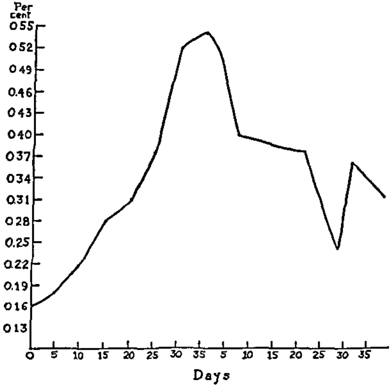


FIGURE 72 Alterations in collagen content in cirrhosis due to carbon tetrachloride The drug was discontinued on the 35th day Reprinted by permission from Morrione, T G *Am J Path* 25, 273 (1949)

This would correlate with a theoretical geometric progression in the number of fibroblasts developing In other words, it takes time for them to start multiplying, and once there are enough of them, the amount of connective tissue which can be formed by them is much greater

In this group we started with a different normal level — 0 16 per cent — arrived at with six animals This lower figure was obtained with a technique which was modified from that originally proposed by Lowry, which we first used and in which an alkaline extraction is the first step Tenth normal sodium hydroxide is added to the liver sample, and the specimen is ground, resulting in viscous solution, at times like a tenacious mucoid jell One has trouble working with it since protein extraction may not be as complete subsequently, even though it is repeatedly submitted to alkaline extraction We have modified the pro-

cedure and use water as the first extraction, followed by a short alkaline extraction, and then a subsequent longer extraction

In other words, it may be that the first "normal figure" of 0 23 per cent included some extraneous protein Perhaps this one does, too, but in this series we seem to be approaching closer to a purer, if you will, collagen

Animals from the original pool were sacrificed at intervals to determine the rate of decrease, some were placed on special diets to see how this fall was affected by special diets, in others, portal vein ligation was performed

The sharp dip between *twenty-five and thirty days following* cessation of the carbon tetrachloride may not be accurate, because it repre-

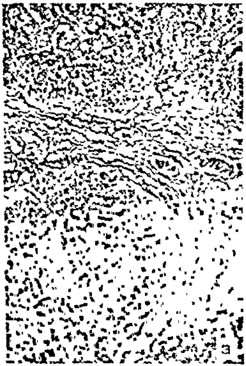


FIGURE 73 Cirrhosis due to exposure to carbon tetrachloride for 30 days Fibrillar collagen and reticulum form broad septa Laidlaw's reticulum and van Gieson's stains × 200 Reprinted by permission from Morrione, T G *Am J Path* 25, 273 (1949)

sents only the arithmetic mean of three animals. Keep in mind the final value of 0 32 per cent, which indicates that the reversal was not complete thirty-five days after exposure to carbon tetrachloride had been stopped

In Figure 73 we have an ordinary reticulum stain of an animal exposed to carbon tetrachloride for thirty days

Figure 74 shows what we observe in a typical instance of reversal of cirrhosis Referring again to Figure 73 it may be seen that there is a typical broad trabecula of reticular material, representing many layers In this animal, cirrhosis was present at thirty-five days, and the chemical value was elevated

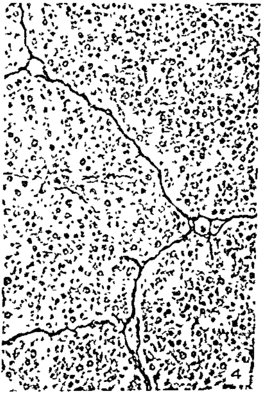


FIGURE 74 Reversal of cirrhosis 30 days after discontinuing carbon tetrachloride There is considerable disappearance of collagen and reticulum, with marked narrowing of the connective tissue septa as compared to Figure 73. Laidlaw's reticulum and van Gieson's stains. × 200 Reprinted by permission from Morrione, T. G, *Am J. Path.* 25, *273 (1949)*

As we tried to follow the removal of this reticular material, all that we observed under the microscope was a progressive thinning and dissolution of the substance The pseudolobules increased in size, as these trabeculae narrowed, and the first impression is that this regenerating nodule of liver tissue exerted mechanical pressure

Figure 74 shows that it is easy to believe that that material was condensed, and yet we know that the chemical values show a significant decrease in the amount of material present

Hartroft: In the preceding Figure are there any small, atrophic but perhaps viable, parenchymal cells in the spaces between the deeply staining reticulin fibers?

Morrione: I think not You may find a few here and there, but most spaces do not contain them

Hartroft. I wonder if it is possible that any atrophic cells which were present might have reverted to normal when the noxious stimulus had been discontinued

Morrione: Yes, I think that does occur to some extent But most of the replacement of the parenchyma is from this aspect of the pseudolobule.

Hartroft: Did you see any evidence of mitotic proliferation in these regions?

Morrione: There is some, I have not seen any in these cells It would seem that the functional state of the liver cells may be related to mechanical compression from these deposits of connective tissue, and as well to the elaboration of the substance connected with the regression of the connective tissue With the ordinary microscope in the cirrhosis which is progressing (at about twenty days), the fibroblastic nuclei are relatively active in appearance Figure 75 shows an animal in which the drug had been discontinued for a month, and here I think there is significant shrinkage. I do not think this is artefact due to overstaining the nuclei of other cells are well stained

We tried to influence this reversal of cirrhosis, or disappearance of connective tissue, with various factors One of the things we tried was diet Within the original group of animals, one hundred were placed on special diets Five different diets were used, twenty animals in each group One of the diets that gave interesting results was a low-protein, high-fat diet The protein content was only 4 per cent (casein) The fat content was 38 per cent (crisco) (See Figure 76)

That diet, in itself, is capable of damaging the liver and inducing cirrhosis, but it was given to the group of twenty animals for only the thirty-five day reversal period, within this group, the cirrhosis persisted In a representative section from an animal in this group, persisting



FIGURE 75. Active proliferation of fibroblasts and bile duct epithelium in hepatic cirrhosis due to exposure to carbon tetrachloride for 20 days The larger cells of a bile duct at the lower left corner are distinguishable from the plump, irregular, ovoid, fibroblastic nuclei elsewhere Hematoxylin and eosin stain. × 1,000 Reprinted by permission from Morrione, T G *Am J Path* **25**, *273 (1949)*



FIGURE 76 Cirrhosis due to carbon tetrachloride 14 days after stopping the drug The fibroblastic nuclei, in comparison to those seen in Figure 75, are shrunken and pyknotic Hematoxylin and eosin stain × 1,000 Reprinted by permission from Morrione, T G *Am J Path* 25, 273 (1949)

cirrhosis can be seen at the end of thirty-five days, and the chemical value averages 0 48 per cent Since all the groups on special diets presumably started with a level of 0.52 per cent (if we accept our sam- [illegible] value at the end of thirty-five days [illegible] t, the difference is not significant On a normal diet, as we saw before, the value for collagen may fall to 0 32 per cent.

To test further the idea that the functional state of the liver cells is important in the removal of this substance, we ligated the portal vein in another group of twenty cirrhotic animals. Adhesions forming between the liver and the surrounding structures served as a complicating factor, and we developed a Talma-Morrison effect (See Figure 77).

The chemical values within this group were not significantly different, except for one animal that had a successful operation with no adhesions The final chemical value, after thirty-five days of reversal, was 0 52 per cent, which is a demonstration of significant impairment in one animal only of reversal of cirrhosis by hepatic vein ligation.

The rationale behind this was primarily the demonstration by Mann that regeneration of liver cells following partial hepatectomy is inhibited by Eck fistula, so that presumably we had inhibited regeneration of liver cells

In order to control the effect of portal vein ligation alone, we tied off the portal vein in a group of twelve animals and studied the collagen and reticulum within their livers, and found that the values did rise There was a rise in the per cent which is not surprising, since these livers do atrophy, and a condensation phenomenon is no doubt occurring A given sample of liver, let us say 1 gm , will contain much more of the original stroma of the organ since atrophy has occurred. However, I do think that portal vein ligation in itself will cause an absolute increase in connective tissue within the liver. I am not absolutely certain that we have done enough on this to establish it, but on the basis of twelve normal animals, with a decrease in liver weight which was not very striking, the collagen content did rise—was actually doubled Except for the normal value in this series of 0 16 per cent, this group showed an average of 0 35 per cent, which is better than double the increase; and yet the liver did not shrink to half the size; it shrunk considerably less That is, the total hepatic collagen then would seem to be increased

FIGURE 77. Increased reticulum 42 days following ligation of the portal vein in a normal rat. Collagen content was 0 35 per cent Laidlaw's reticulum and van Gieson's stains. × 500 Reprinted by permission from Morrione, T G *Am. J. Path.* **25**, *273 (1949).*


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7

This is, of course, an instance of experimental cirrhosis, and only one instance of newly-formed connective tissue. One other instance in which resorption of connective tissue may occur was mentioned yesterday, the pregnant uterus Perhaps, too, in the healing of fractures there is deposition of fibrous tissue in the early phases, and that is removed and replaced by bone, although there we may be getting into an argument of transformation I do not know how Dr Bennett feels about that, whether the connective tissue is transformed into bone, or would you say that it has to be removed first?

Bennett I have not given much thought to callus in relation to this problem However, on the spur of the moment, I would think that in the case of fracture repair, the callus is probably actively removed through a process of dissolution of matrix of bone, and simultaneous removal of the inorganic salts—a process of actual breakdown and removal

Dr Angevine has studied this problem more particularly. Would you agree, Dr Angevine, that this is a different process from the one under discussion?

Angevine. Of course, you are getting at the nature of what osteoid is and I do not think I am qualified to say exactly what it is Such a discussion would open up another subject I have never felt that bone and subsequently mineral salts are deposited directly in connective tissue Some special transition must occur first in the connective tissue

Bennett: In the removal of the excess callus, do you feel that there is actual resorption of the matrix with simultaneous removal of bone salts?

Angevine. Membranous bone forms right in the callus, and then I suspect that the callus disappears, as you have indicated But what the degree of incorporation is, and what the degree of disappearance is, would be an entirely new problem and would have to be attacked in much the same manner as it has been worked out in the liver.

Morrione: There is one point we thought of. Inasmuch as we were observing movement of this protein, we might radically regard the collagenous protein within the liver as a storage protein In other words, being quite liberal about our concepts, we thought that possibly a low protein diet, for example, might make increased demands upon this collagenous protein within the liver

I have analyzed the data we have, which includes one group of animals on a low-protein diet, and interestingly enough, the amount of reversal of cirrhosis seems to be a little better on a low-protein diet supplemented with methianine, choline, and cystine, than on any other diet

Our values are 0 28 per cent as compared to 0 32 per cent I do not think that is very striking, and the sections do not show too much difference I certainly do not think we have begun to prove or disprove, that this can be utilized as a storage protein. I have brought it in as a thought inasmuch as I do think it is a labile protein, a foreign one at that, with a rich complex source of amino acid

Bennett: Some of your earlier photographs gave me the impression that the architectural design of the liver was a bit changed, that the lobular pattern was cut up a bit as we see it at times in human cirrhosis Is this true?

Morrione: Yes, it is

Bennett: What is your impression concerning reversal toward the normal lobular pattern with diminishing amounts of connective tissue? Is that pretty well restored eventually?

Morrione: Remarkably well, Dr Bennett I do not know how it occurs I have not followed it through those stages, but you do end with a liver remarkably similar to the normal state The lobular pattern is very well reproduced, although there is some distortion, probably because we have observed a more incomplete reversal than complete

Porter: What does this heavy deposition of collagen achieve for the liver?

Morrione: In what respect?

Porter I notice that the lobes retain their shape in the heavily cirrhotic liver with carbon tetrachloride

Morrione: The lobes are maintained, but the lobular pattern is not I am not even certain, Dr Porter, that we have reparative fibrosis In other words, in the pathogenesis of this lesion, the classical explanation is that there is necrosis, and that the necrosis is the stimulus for fibroblastic proliferation, and that is the sequence of events

From what I have been able to observe personally on the pathogenesis of cirrhosis, necrosis is conspicuous by its absence as a stimulus

Hill: Is this experimental or human?

Morrione I think it is in all cirrhosis, experimental and human

Ragan: In these animals, do you see any invasion of wandering cells before the appearance of fibroblasts?

Morrione. There are many macrophages present

Angevine: In the liver, prior to the formation of the tumor following administration of butter yellow, there is demonstrable necrosis of liver cells prior to regeneration That is before the collagen appears I would suspect that in carbon tetrachloride poisoning, there must be considerable destruction as well, prior to the regeneration

Bennett: With an appropriate single dose of carbon tetrachloride, one can destroy liver cells in the central portion of the lobules, and if one uses a reticulum stain at that time, one can stain the framework which seems to be the framework of the liver lobule minus the parenchymal cells which have become necrotic and removed In similarly treated animals, killed twenty-four to seventy-two hours later, one can see almost complete regeneration of liver cells without any demonstrable—speaking of microscopic appearances now—increase in connective tissue Is that not your finding?

Morrione: Yes.

Bennett. Presumably then you are showing us livers that are changed by a more persistent type of injury, and presumably at a slower rate of cell destruction and cell regeneration.

Morrione And we do have fibroblastic proliferation together with it That is true

Campbell: In general, it is often difficult to catch the liver in the necrotic state. In a disease like Weil's disease, where the liver may be full of mitoses when the patient dies, you may find it difficult to find a cell you can regard as a necrotic cell One presumes that some have undergone necrosis, because there are mitotic figures, also from the fact that the liver cells generally are swollen, and that correlating the total weight of the liver with the average assessed size of the liver cells microscopically, there appear to be fewer cells in that liver than in the normal liver

It is difficult to find the actual necrotic cells. It seems to me that once they have decided to die, [illegible] ed

Bennett. I think they [illegible] but occasionally it is possible to [illegible] you see necrotic cells, or cell fragments that are infiltrated or invaded by leukocytes showing that they are necrotic.

Campbell But the fact that you do not see necrotic cells in your experiments, Dr Morrione, is not a guarantee that there has not been a lot of necrosis

Maegraith: What is the distribution of this fibrous tissue in relation to the lobular pattern?

Morrione: In the early stages, it is central-lobular and peripheral Later it assumes a haphazard form with pseudolobule production As you saw, there were these varied sized nodules

Maegraith. But they were not necessarily the original lobules just regenerating They were bits.

Morrione· Yes, pseudo

Maegraith· I thought in carbon tetrachloride poisoning, the cells affected first were in the mid-zonal region where a hydropic change

develops. If that is the case here, where you are dealing with minimal degeneration, I would expect your lesion to develop there, rather than in the central region of the lobule where the massive necrosis develops

served.

Maegraith I think if you look at your animals early, you will find that the cells which are chiefly affected, the cells which are affected first, are those in the mid-zonal region, and that the swelling of these, as Himsworth has shown, causes obstruction to the blood flow, and following that, you get your central necrosis I think that it proceeds in that order

Hartroft: In experiments conducted in our laboratories the results were not strictly in agreement with those just described by Dr Maegraith. Small doses of carbon tetrachloride in oil were administered by subcutaneous injection to Wistar rats weighing 100 to 125 gm The animals were maintained on a normal, stock diet and killed at daily intervals Ordinary paraffin sections of the livers were prepared and stained by the usual methods for demonstrating connective tissue, etc In addition, thick (100 micra) slices of livers, which had been injected with India ink, were cleared with benzyl benzoate The initial lesions were always restricted to the region of the liver lobule immediately surrounding the central veins. The first change consisted of severe hydropic swelling of the parenchymal cells in these areas, followed later by their coalescence, rupture and lysis

Maegraith: This is one of the points that these conferences bring up so clearly, because I have no doubt whatever in our experiments that the lesion does start in that mid-zonal group of cells *

Angevine: Many times one is given a liver section, especially from an animal, and is asked to determine whether a lesion is mid-zonal, and the answer is very difficult to give

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*Since the time of the Conference, Professor Maegraith and his associate, Dr W H H Andrews have discussed these apparent discrepancies with Dr Hartroft in more detail Dr Maegraith

by stomach tube It is quite possible that absorption by the latter route might not be nearly as complete as by subcutaneous injection The Liverpool and Toronto groups agree that the most reasonable explanation at hand for the apparent differences in their results may be connected with the discrepancies in the amounts used and method of administration of the active substance —*Editor*

Bennett A number of years ago, with Dr Cecil Drinker, we performed some experiments in which the object of the study was to test *the toxicity of certain chlorinated hydrocarbons* (3) These compounds were administered to rats in a variety of ways and in varying doses It was observed that although animals on certain dosages seemed to remain in good health, their livers became swollen and mottled On histologic examination, the parenchymal cells were tremendously swollen and contained either hyaline masses within the cytoplasm, or vacuoles When such animals were given orally small doses of carbon tetrachloride, they were exceedingly susceptible to the drug Doses of carbon tetrachloride which were sublethal in the normal animal, would kill most, if not all, of the experimental animals that had been exposed to the chlorinated hydrocarbons

Although the pre-existing changes in the liver seemed to be distributed similarly in all cells throughout the lobules, it was the central portion of the lobules that became necrotic following administration of carbon tetrachloride.

From these observations on the comparative effects of carbon tetrachloride on previously damaged and normal livers, it would seem that the central portions of the liver lobule are more vulnerable to a hepatotoxic agent. This may have to do with the vascular supply of the lobule

Maegraith I agree about the central lobular necrosis What I said was that in rats the early hydropic changes appeared first in the midzonal cells We feel that in carbon tetrachloride poisoning the swelling of these cells produces stagnant anoxia in the center of the lobule, which gives rise to necrosis

Hartroft. Dr *Morrione suggested the possibility that the cirrhosis* produced by carbon tetrachloride administration receded more quickly if the animals were fed a diet low in protein than if they were given a diet containing a high percentage of protein, providing the carbon tetrachloride was withdrawn in both instances. In agreement with Dr. Morrione's other findings are those of Sellers, Lucas and Best (4) at the University of Toronto Their experiments demonstrated that recession of the fibrous tissue in cirrhosis produced by carbon tetrachloride administration varied with the lipotropic content of the diet when all other factors were kept as constant as possible. Their results confirm those of Dr Morrione's which showed that the cirrhosis regressed poorly when the animals were fed a high-fat, low-protein diet for in such a food-mixture the lipotropic content would be low. Although biochemical determinations of liver-collagen were not performed in the experiments of Sellers, Lucas and Best, the quantitative estimations of fibrous tissue made from examination of histological material, agree

very well with those reported by Dr Morrione

Hill In my experience in ordinary cirrhosis, if you do a silver stain, you have thin fibers, but if you stain with the ordinary connective tissue stains, the fibers appear broader and much more extensive I found with good sublimate fixation, that one obtains good results.

Morrione: But you do not see reticulum at all The reticulum is completely missing from your vision We have to be careful here, because we are talking about two substances The ideal stain would be a silver stain, counter-stained with trichrome We have done that, but those preparations, unfortunately, come out too dense, at least in our hands Dr Wolbach utilized that in some of his original studies on scurvy, and he has some nice illustrations of a trichrome superimposed upon a reticulum stain (5)

Campbell· You have been working on paraffin sections?

Morrione: Exclusively

Campbell: In my experience, on the liver and elsewhere, a silver impregnated frozen section gives you not only a much more esthetically pleasing effect, but you have a three-dimensional picture of things because of the thicker section If you leave it untoned, you get black reticulin fiber staining, and golden yellow-brown collagen staining You can see a much more complete picture of connective tissue fibers in general I wonder if that might not help in demonstrating the amount of collagen in your sections

Morrione· During this reversal phase, we were looking for alterations in fibers with our crude techniques, and I will say this tinctorial changes can be observed in these fibers I am not at all convinced, however, that they are significant, but fibers are seen during this reversal phase that show variation in staining, and one has the impression—purely an impression—that some change is taking place within the fibers

I think Dr Porter has the only answer for it If we could see this breakdown actually occurring, and, could establish the appearance of the breakdown for instance under the influence of an enzyme, we would really be making progress

Hill I think, actually, I probably misunderstood you I would like to show three illustrations of our viewpoint Figure 78 shows a condition which we call "collagenosis" In the next section which is stained for reticulin the lesions are quite clearly shown (See Figure 79) You can see in Figure 80, a modification of the Mallory stain, that the lesion appears to be much more extensive, and the blue stains quite heavily, more than would be seen in the actual reticulin stain

Morrione. I think the trichrome stain for collagen has very limited value If you attempt to judge the extent of cirrhosis, based solely on

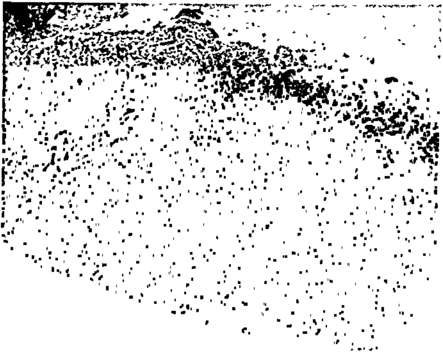


FIGURE 78 Eosinophilic coagulum deposited in several areas of lobules from liver of child aged 2½ years H & E × 60



FIGURE 79 Next section to Figure 78 stained for reticulin with modified Foot-Wilder stain Note the reticulinosis in areas showing eosinophilic coagulum in Figure 78 × 60



FIGURE 80 Next section to Figure 79 stained with modified Mallory connective tissue stain [illegible]
philic coagulum in Fi [illegible]
graph does not show [illegible]
connective tissue stain are much coarser and broader than the fine staining fibers of the reticulin stain

a trichrome stain, you are handicapping yourself tremendously I do not say that it does not demonstrate collagen, but it certainly does not show reticulum, and in my opinion the changes in reticulum are frequently much more striking than in collagen

Hill But, when the collagen is aging—of course we are on debatable ground here—some of the fibrils may lose their properties, but they are stained by the trichrome much more extensively

Morrione: You should utilize both

Hartroft. Did you find, Dr Morrione, that during the recovery stages of carbon tetrachloride-cirrhosis it was difficult to obtain intense blue-staining of the trabeculae? This was our experience, despite repeated attempts to obtain deeper stains for the purpose of color photography

Morrione Dr Hartroft's point raises an interesting side issue—that human cirrhotic livers appear beautifully stained with trichrome That is, the demonstration of blue-staining material in human cirrhosis is considerably better than in this experimental material

Whether we are looking at later stages with more trichrome-stainable material in the human cases, as compared to the fresher cirrhosis and more reticulum-rich material here, I really cannot say However, it is a fact that I have not been able to see the beautiful trichrome preparations—by that I mean the broad, blue septi—in experimental cirrhosis that are seen in human cirrhosis

Ragan Does the rat have any peculiarity in respect to collagen formation?

Morrione. Not as far as I know

Hartroft. Could the explanation be a matter of degree and age? In most human material, the collagen has probably been present in the liver for a long time before death

Ragan If you section a wound in a rabbit or a human, trichrome stainable material is seen in five days

Hill. There is one other thing Remember, the pathologist never thinks that what he sees through a microscope might be like some of the rats in which reversibility is taking place

If reticulin fibers with very little blue-staining collagen are present, you will say it is an early stage progressing, not that it is regressing

Lansing Does not the condition of the tissue determine to some extent the tinctorial properties not only of collagen but other materials? The usual autopsy material that you deal with is obtained at least six, eight, and often tweny-four hours after death Quite a few postmortem changes are going on not only in collagen, but elsewhere

When you take tissue from an experimental animal, it is usually minutes after death, if the animal is dead at all It may well be, for

example, that there are many oxidative changes going on in collagen, and as I pointed out yesterday, oxidation will greatly enhance the tinctorial properties of many materials, and especially collagen.

Ragan: We can see beautiful tinctorial qualities of biopsy wound taken from a living animal.

Lansing: I do not think one can compare collagen in a twenty-four-hour postmortem tissue with freshly obtained tissue.

Ragan: It looks the same tinctorially

Hartroft: In our animal material we have found that tinctorial reactions are very much poorer if appreciable postmortem change has developed

Lansing: It is a matter of degree, I am sure

As I commented yesterday, if one oxidizes a piece of tissue containing collagen as well as elastin, one sees beautiful staining with the usual elastic stains of fibers that are definitely collagenous, ordinarily refractory to the Verhoeff, resorcin-fuchsin or orcein stains They are beautiful, but definitely collagenous Pathological material is difficult to interpret

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*These references were supplied by Dr Morrione but not given in the text —*Editor*